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Chromatographic Studies of Nucleic Acids

4. THE NUCLEIC ACID OF THE TURNIP YELLOW MOSAIC VIRUS, INCLUDING A NOTE ON THE NUCLEIC ACID OF THE TOMATO BUSHY STUNT VIRUS

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(*Received* 23 *November* 1950)

It was largely because of our interest in the turnip yellow mosaic virus (Markham & Smith, K. M., 1949) that we began our study of nucleic acids. This virus contains about 37% by weight of ribonucleic acid, which is an unusually high proportion. All the infected plants which we have examined contain, as well as the virus, another characteristic and related protein in an amount equivalent to 25–30% of the weight of the virus nucleoprotein. This protein, as far as we know, cannot be separated from the virus by any method other than fractional centrifuging, as the two proteins differ very little, if at all, in their solubilities in various solutions, in electrical charge and in their size, shape and general surface properties. This second protein appears to be the virus nucleoprotein minus its nucleic acid, and it is non-infectious. It is a very poor antigen in rabbits, although it combines readily with antiserum to the nucleoprotein, which itself is an excellent antigen. In these two properties, ability to cause a disease and ability to cause antibody production in rabbits, we would appear to have biological activities associated with a nucleic acid, and with a view to obtaining further information about the function of nucleic acid in viruses we have undertaken the analysis of several virus nucleic acids.

We describe in the following paper several refinements of our earlier techniques and we have confirmed most of our findings by orthodox micro-analytical procedures, which, combined with chromatographic methods, can be used to analyse ribonucleic acids without the need for an ultraviolet spectrophotometer.

METHODS AND MATERIALS

Precautions necessary for maximum accuracy

When we began our investigation of nucleic acids it seemed that an error of $\pm 5\%$ or thereabouts could be tolerated, and such a figure may be obtained without a great deal of trouble by the method which we have already published (Smith, J. D. & Markham, 1950). However, as we checked the various sources of variability in our methods, it became evident that considerably greater accuracy was possible, and as small variations in the compositions of the nucleic acids of plant viruses may be of importance, we considered it worth while to improve our methods.

Much of the inaccuracy when dealing with small quantities of materials is due to systematic volumetric errors, and in this investigation we have used throughout one capillary pipette having a volume of 14·0 µl., both for measuring out the solutions to be put on the chromatograms and for taking samples for P, N and sugar analysis. This simple precaution eliminates most of the systematic error.

It is obvious that extreme care should be taken in calibrating the cells used for spectrophotometry before making each set of readings, and, although tedious, it is a good plan to read all the solutions including the blanks in one cell against a water blank in the other cell. A considerable source of error was traced to the test tubes used for extracting the spots. Traces of commercial detergent give rise to measurable densities in the ultraviolet and are difficult to remove completely. In consequence we have cleaned all test tubes used with a few drops of ethanol followed by conc. HNO_3, rinsing after the violent reaction with tap water and then distilled water.

The last main limiting factor in the spectrophotometric estimation is the paper blank reading which is generally between 0·01 and 0·05 density units, increasing from the top of the sheet to the solvent front. This variation may be made negligible by the use of large quantities of nucleic acid hydrolysate, and it is in this that chromatographic solvents containing strong HCl show their superiority, as they can move 1–2 mg. of nucleic acid hydrolysate without any sign of 'tailing'. There would, however, appear to be no advantage in increasing the quantity of nucleic acid in each spot above 1·5 mg. as this amount gives densities of at least 1·0–2·0 in the individual extracts in 5 ml. of 0·1 N-HCl. It is very striking that when replicate spots in this density range are read they usually do not differ by more than $\pm 0{\cdot}002$ unit. When working in this range, the correction for the volume occupied by the nucleic acid in solution becomes important if the results have to be referred to a dry weight basis, being of the order of 5–10%. The correction may, of course, be determined experimentally by measuring the P content of the hydrolysate, but the approximate correction which we have given previously should be adequate for most purposes. The minimum amount of fluid for the hydrolysis should be 0·05 ml., as below this level the volume change due to the saturation of the air space in the hydrolysis tube may become limiting.

With the refinements given above, the overall accuracy is such that it becomes necessary to take into account the hydrolysis of the pyrimidine nucleotides. We have determined the extent of this hydrolysis experimentally as being of the order of 5%, and this correction may be applied without determining it for each hydrolysis.

Preparation of virus nucleic acid

The nucleic acid of the turnip yellow mosaic virus is liberated from the latter by treatment with 30% or more (v/v) of ethanol in neutral solution at room temperature (Markham & Smith, K. M., 1949). Several preparations have been made by this method from crystalline virus. Subsequent treatment involved removal of proteins by centrifuging, and by the method of Sevag, Lackman & Smolens (1938). The material was used in an 'air-dry' condition and probably contained a considerable amount of water. Tests for protein were negative.

Analytical methods

P was estimated throughout by Allen's (1940) method. Total P and the P bound to pyrimidines were estimated by incinerating with 60% (w/v) $HClO_4$, while inorganic P was estimated without further hydrolysis, either from pipetted volumes of liquid or on extracts of filter-paper spots made in dil. $HClO_4$. The incineration of spots cut from developed chromatograms proceeds fairly smoothly, and we have only had a small number of the incinerations explode. It is, however, wise to take the precaution of wearing some protection for the eyes when near the digestions. The blank spots cut out as controls were estimated at about 0·5 μg. P so that quantities of some 25–50 μg. of bound P could be estimated with fair accuracy. In the chromatograms run in solvent 3 (see below) the blanks tended to be rather higher and more variable than with the other solvents.

Inorganic P was estimated from spots on the chromatogram by finding its position by the method of Hanes & Isherwood (1950) and then cutting out duplicate spots from parallel lanes. The spots were then extracted overnight in 10 ml. of water, washed out and made up to volume with the reagents used for the P estimation.

The absorbing spots on the chromatogram were found by our usual method (Markham & Smith, J. D., 1949) in some cases with a modified filter system (Markham & Smith, J. D., Appendix). We have also used the fluorescence method of Holiday & Johnson (1949) (see also Carter, 1950), viewing the chromatograms through a violet filter (e.g. Wratten C_5). But we have found it impossible in some cases to see any guanylic acid, apparently because the fluorescence of the latter matched that of the filter paper.

The other analytical procedures used have been described previously (Smith, J. D. & Markham, 1950). The molecular extinction coefficient for guanine which we now use is slightly different from that used previously ($\epsilon \times 10^{-3}$ at 250 mμ. in 0·1 N-HCl = 11·0). This value, obtained by Wyatt (1951) and confirmed by us, is 4% higher than our earlier figure.

Hydrolysis methods

Two methods of hydrolysis have been used. Method (i), using N-HCl at 100° for 1 hr. (Smith & Markham, 1950), liberates adenine, guanine, cytidylic acid, uridylic acid and small amounts of cytidine and uridine (about 5% of the pyrimidine nucleotides are hydrolysed to nucleosides).

Method (ii) is hydrolysis with N-NaOH or N-HCl at room temperature, which yields a solution containing the nucleotides of the four bases. N-NaOH is the better of the two as ribonucleic acid becomes sticky in N-HCl and it takes a long time for solution to take place. As a routine we place the nucleic acid in a small serum bottle, add the NaOH and leave 18 hr. or more before using. No further hydrolysis takes place so that the solution may be kept for weeks. The solution is put on to the papers and if acid solvents are used need not be neutralized. On the other hand, with solvent 3 it is necessary to neutralize the spot on the paper with a small excess of N-acetic acid and to dry before running the chromatogram. If N-HCl is used for the hydrolysis, it is important that the spot should not be allowed to dry, as in this process the acid increases in strength and further hydrolysis of the nucleotides may take place.

Chromatographic solvents

For chromatography of the mixtures obtained by hydrolysing the nucleic acid in several ways we have used three solvents. Solvent 1 is 7·45 M-*tert.*-butanol, 0·8 N-HCl in water (Smith, J. D. & Markham, 1950). Solvent 2 is 8·5 M-*iso*propanol, 2 N-HCl in water (Wyatt, 1950). These two mixtures are similar in their ability to resolve the various bases, but differ in that no. 1 resolves uridine while no. 2 resolves cytidine, thus permitting the estimation of the traces of these nucleosides which are present in hydrolysate (i).

As in the case of most chromatographic solvents consistent R_F values may only be obtained with these mixtures by taking elaborate precautions such as maintaining constant temperatures and exact maintenance of solvent composition, etc. The relative positions of the substances, however, are reasonably constant and it is possible to identify most spots on chromatograms by inspection. Consequently we have been in the habit of using hydrolysates of nucleic acids of known composition as markers, as such markers give much more information than spots of single substances. Fig. 1 gives an indication of the positions of the spots on chromatograms run in these solvents. Only uridylic acid is resolved in either solvent from the mixture of nucleotides produced by cold acid or alkaline hydrolysis (Fig. 1 *B*).

Solvent mixture 3, which we developed for separating the purine nucleotides from the mixture produced by cold alkaline hydrolysis, differs greatly from solvents 1 and 2. It consists of saturated $(NH_4)_2SO_4$, 79%, water, 19%, and *iso*propanol, 2% (v/v/v). A buffer solution (0·1 M, pH about 6) may with advantage be substituted for the water, but it is not essential. It may also be found advantageous to have a small excess of *iso*propanol in the solvent in the bottom of the tank. This solvent runs rapidly, 6 hr. being the usual time for running 30 cm.

Commercial yeast adenylic acid gives two spots when run in this solvent, the one with the larger R_F value (adenylic acid *a*) running slightly behind muscle adenylic acid and comprising about 25·6% of the total (this value is only a characteristic of the method of production of this adenylic acid). From this it would appear that adenylic acid *a* is the same as the adenylic acid *a* of Carter (1950), who originally found that yeast adenylic acid was a mixture of two related substances. The more slowly moving substance, constituting 74·4% of this specimen of commercially purified yeast adenylic acid, separated in solvent mixture 3, presumably corresponds with

Carter's adenylic acid *b*. In hydrolysates of ribonucleic acids these two adenylic acids are also found, and in addition two guanylic acids (also named *b* and *a* in order of increasing R_F value) are resolved on the chromatogram while the uridylic and cytidylic acids

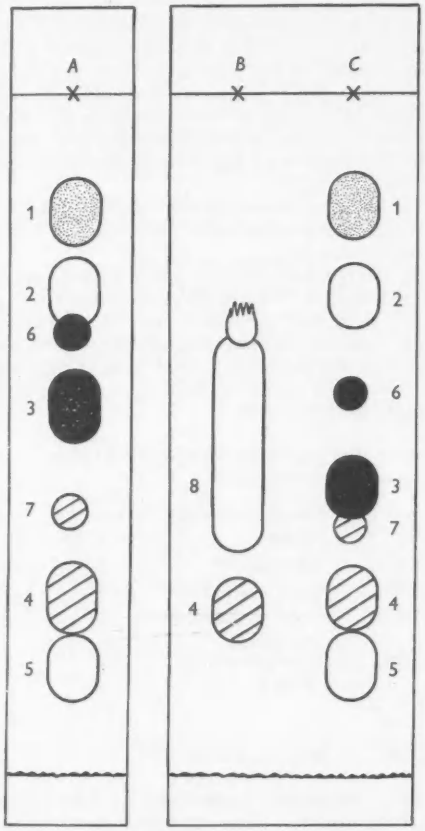

Fig. 1. Chromatograms of hydrolysates of ribonucleic acids. *A*, hydrolysed in N-HCl, 1 hr. at 100° run in solvent 1; *B*, hydrolysed in N-NaOH at 20° run in solvent 2; *C*, hydrolysed in N-HCl, 1 hr. at 100° run in solvent 3. The spots are: 1, guanine; 2, adenine; 3, cytidylic acid; 4, uridylic acid; 5, H_2PO_4; 6, cytidine; 7, uridine, and 8, a mixture of adenylic, guanylic and cytidylic acids.

are not resolved from each other (Fig. 2). A disadvantage of this solvent is that it is unable to move large quantities of the nucleotides and so it is necessary to spread out the hydrolysate of 1 mg. of a nucleic acid over 5 cm. at the top of the filter paper instead of putting it on as a compact spot.

RESULTS

For the purposes of comparison we have converted all the analytical results into μmol./mg. dry weight of the starting material. This procedure has the advantage that all the values may be compared

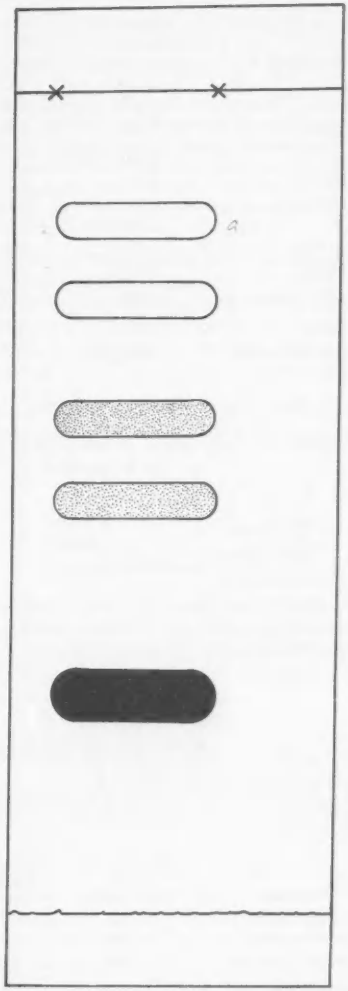

Fig. 2. Relative positions of nucleotides in solvent 3. From top to bottom: □, adenylic acids *b* and *a*; ▤, guanylic acids *b* and *a*, and ■, cytidylic + uridylic acid.

directly. It is surprising that this method of presentation is not adopted more generally in making up balance sheets of complex substances such as proteins.

Table 1. *Composition of turnip yellow mosaic virus nucleic acids as determined by ultraviolet light-absorption measurements*

(The two sets of figures refer to analyses from the nucleic acids of two entirely different virus preparations. The figure in brackets is the number of sets of determinations.)

Substance	Preparation 1 (4) (μmol./mg.)	Preparation 2 (3) (μmol./mg.)
Adenine	0·616	0·60
Guanine	0·460	0·44
Total purines	**1·08**	**1·04**
Cytidine	0·06 (±1·8%)	—
Cytidylic acid	0·98	1·01
Total cytosine	1·04	1·06
Uridine	0·02	—
Uridylic acid	0·59	0·58
Total uracil	0·61	0·61*
Total pyrimidines	**1·65**	**1·67**
Total bases	**2·73**	**2·71**
Weight of nucleotide residues	866 μg./mg.	

* Determined on alkaline hydrolysate.

Hydrolysates prepared from turnip yellow mosaic virus nucleic acid were analysed in the following way. Adenine, guanine, cytidine, cytidylic acid and uridylic acid were estimated spectrophotometrically after chromatography of the hydrolysate in solvent 2. Uridine in the hydrolysate was estimated after chromatography in solvent 1. The results are presented in Table 1. The standard errors were only calculated for cytidine as the maximum difference observed in the measurements of the purines and the pyrimidine nucleotides was 0·7%. The total of the bases accounts for 102% of the phosphorus in the nucleic acid if one assumes one atom of phosphorus per molecule of base. This value is not significantly greater than the expected 100%.

The following substances were estimated:

(1) Total P was estimated on samples of hydrolysates (i) and (ii).

(2) Uridylic acid P from spots of hydrolysate (ii) run in solvent 2. This analysis gives the total uridylic acid P, as in this hydrolysis no uridine is formed.

(3) Cytidylic acid + uridylic acid P was also estimated in the same hydrolysate from spots run in solvent 3, and hence by subtraction the cytidylic acid P was determined.

Table 2. *Composition of turnip yellow mosaic virus nucleic acids with their standard errors as determined by normal micro-analytical techniques*

Substance	μmol./mg.	Substance estimated	No. of estimations
Total purines	1·07	Pentose	2
Total purines	1·03 (±2%)	Inorganic P*	6
Uridylic acid	0·58 (±1·6%)	Uridylic acid P†	3
Total pyrimidines	1·67 (±1·7%)	Total pyrimidine P	3
Cytidylic acid	1·09	By difference	—
Cytidylic acid	1·07 (±1·4%)	Cytidylic acid P†	5
Total bases	2·72	Total P	4
Total bases accounted for by purine P + pyrimidine P	2·70	—	—

* 5% of the total pyrimidine P subtracted. † Value found × 1·05.

Table 3. *The ratios of the nucleotides in turnip yellow mosaic virus nucleic acid calculated to an arbitrary total of* 4·00

Substance	Molar ratio from absorption	Molar ratio from N estimation
Adenylic acid *a*	0·51*	—
Adenylic acid *b*	0·40*	—
Total adenylic acid	**0·91**	0·88
Guanylic acid *a*	0·35*	—
Guanylic acid *b*	0·34*	—
Total guanylic acid	**0·69**	0·75
Cytidylic acid	1·53	1·46
Uridylic acid	0·89	0·91
Purines/pyrimidines	0·65	—

* Obtained from the ratios of the two extinctions and the total purine found.

(4) Cytidylic acid P was estimated in hydrolysate (i) using solvent 2. This value may be corrected to a close approximation to give the total cytidylic acid P (including that originally associated with the cytidine) by multiplying by 1·05, a value deduced from the relative absorption of these substances when extracted from the chromatogram.

(5) The phosphorus associated with the purines + the pyrimidine nucleosides was estimated on the whole hydrolysate (i) and also on the spots of phosphoric acid from chromatograms run in solvent 2. The results in both cases were identical. Using a minor correction for the P liberated from pyrimidine nucleotides (approx. 5% of the total pyrimidine) the purine P may be deduced.

(6) Pentose was estimated in hydrolysate (ii). The sugar was identified as ribose by a chro-

matographic method (Markham & Smith, J. D., 1950).

These results are tabulated in Table 2. The molar ratios of the nucleotides calculated to an arbitrary total of 4 are given in Table 3. These values are derived from the figures in Table 1.

Nitrogen estimations were also made on spots cut out from chromatograms of an acid hydrolysate run in solvent 2. The results showed considerable variation because of the small amounts of purine or pyrimidine nitrogen in the spots (22–53 μg.) relative to the blank in the paper (about 20–40 μg.), but as a matter of interest are given in Table 3 for comparison. The total recovery of nitrogen from the spots was equivalent to 2·64 μmol. of base per mg. nucleic acid, but is, of course, subject to considerable error.

The purine nucleotides

Values are given in Table 3 for the relative amounts of the two isomeric adenylic and guanylic acids which we found in chromatogram spots of hydrolysates run in solvent 3. These spots have been identified by extracting and hydrolysing in N-hydrochloric acid at 100° for 1 hr. and running in solvent 1

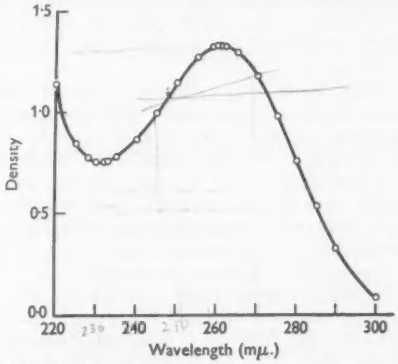

Fig. 3. The ultraviolet absorption spectrum of turnip yellow mosaic virus nucleic acid. A solution equivalent to 4·82 μg. P/ml. in a 1 cm. cell.

or 2. That these double spots are not artifacts may be demonstrated by cutting them out and re-running in solvent 3, when they maintain their relative positions even after further treatment with N-sodium hydroxide. This indicates that they are not an equilibrium mixture formed in alkaline solution, although we have confirmed Doherty's (1950) observation that they are interconvertible in acid solution. While we have not been able to make accurate P analyses on these spots, our values are sufficiently reliable to show that one atom of P is combined with each molecule of purine in these nucleotides. The absorption spectrum of the whole nucleic acid is shown in Fig. 3.

The nucleic acid of the tomato bushy stunt virus

The nucleic acid of this virus has also been analysed, but the method used has been slightly different. The nucleic acid is more difficult to remove from the protein, so we hydrolysed the whole virus.

Virus (4 mg.) was taken in a small test tube in the form of a solution. The virus was precipitated by adding $(NH_4)_2SO_4$ to 1/3 saturation and was centrifuged down. The supernatant liquid was poured off and the tube wiped inside with filter paper. N-HCl (0·05 ml.) was added and the stoppered tube was placed in a water bath at 100° for 1 hr. Most of the protein was insoluble after this treatment and was sedimented by centrifuging. The supernatant liquid was pipetted off on to the filter paper for chromatography. Five 14 μl. spots were run in solvent 2, three of which were then used for spectrophotometric determination of the purines and the pyrimidine nucleotides.

The results are given in Table 4. The other two lanes of the chromatogram were neutralized with ammonia vapour and then sprayed with ninhydrin to check that no aromatic amino-acids were present to interfere. The amount of free amino-acids was very small.

Table 4. *Molar ratios of the bases of tomato bushy stunt virus nucleic acid, calculated to an arbitrary total of* 4·00

Adenine	0·995
Guanine	1·12
Cytosine	0·875
Uracil	1·01
Purines/pyrimidines	1·12

Standard errors were computed from the readings of the densities and were small. The largest of the series was uridylic acid, $\pm$ 1·5 %, which is quite satisfactory in view of the small amount of material used.

DISCUSSION

The figures presented in Tables 1–3 show that it is possible by using certain precautions to analyse a ribonucleic acid with considerable accuracy either by ultraviolet absorption measurements on chromatogram spots or by phosphorus analyses on similar spots in conjunction with other measurements. The former is obviously the method of choice, but it is apparent that in order to take full advantage of the reproducibility of the technique it is necessary to apply minor corrections for the hydrolysis of the pyrimidine nucleotides during the liberation of the purines. As the correction is small and fairly constant, the most convenient method of calculating the results is to use the following molar extinction coefficients:

Guanine	$\epsilon_{250} = 11{\cdot}0 \times 10^3$
Adenine	$\epsilon_{260} = 13{\cdot}0 \times 10^3$
Cytidylic acid	$\epsilon'_{280} = 12{\cdot}3 \times 10^3$
Uridylic acid	$\epsilon'_{260} = 9{\cdot}45 \times 10^3$

The dash signifies that the extinction coefficient is an apparent and not an actual one. Using this set of figures and taking the precautions outlined it should be possible to obtain results within about ±1% on any ribonucleic acid. In this regard it should be pointed out that in cases such as that of the turnip yellow mosaic virus nucleic acid, where large amounts of cytidylic acid are found, it is advantageous to use solvent 2, as the error which may be introduced into the estimation of cytidylic acid by the presence of uridine is very small when readings are made at 280 mμ., while the absorption of cytidine may cause an appreciable if small error in the adenine estimation. Solvent 2, however, needs to be run at least 30 cm. down the paper, but this is to some extent compensated for by its greater rate of flow.

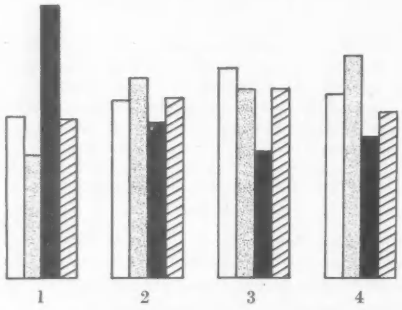

Fig. 4. The compositions of four ribonucleic acids (N.A.) in molar proportions of nucleotides. From left to right turnip yellow mosaic N.A., tomato bushy stunt N.A., tomato mosaic N.A. (Markham & Smith, J. D., 1950) and yeast N.A. (Smith, J. D. & Markham, 1950). The columns are: □, adenylic acid; ▤, guanylic acid; ■, cytidylic acid, and ▨, uridylic acid. (These conventions are also observed where possible in Figs. 1 and 2.)

A fact which emerges from the present analyses of the nucleic acids of turnip yellow mosaic virus and the tomato bushy stunt virus and the previous analyses which we have made, is that the general pattern of these ribonucleic acids is very different (Fig. 4). While it is evident that more analyses of different plant viruses need to be made, the results suggest that the ribonucleic acids may be characteristic of virus 'genera' and thus be useful in the classification of viruses. It is with this point in view that we have started to develop methods of analysis which can be applied to small quantities of whole virus, enabling data to be obtained for viruses which can only be prepared in milligram quantities.

We have given in this paper data concerning the amounts of the isomeric adenylic and guanylic acids in turnip yellow mosaic virus nucleic acid, but feel that their interpretation must await further work. If their properties are found to be characteristic they might prove to be of considerable importance in the study of nucleic acid structure, but if, as seems likely, they are only α and β forms of the nucleoside-3-phosphoric acids as has been suggested recently (Doherty, 1950), it might be dangerous to stress the significance of differences.

SUMMARY

1. Improvement in methods for the chromatographic analysis of ribonucleic acids are described. The overall accuracy is of the order of ±1%.

2. A method for the quantitative analysis of ribonucleic acids by chromatography combined with normal micro-analytical techniques is outlined.

3. Turnip yellow mosaic virus nucleic acid has been analysed by both methods and close agreement has been obtained between the results.

4. Tomato bushy stunt virus nucleic acid has been analysed by a modified technique suitable for application to small quantities of virus material.

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APPENDIX

A Simple Filter Outfit for the Short-wave Ultraviolet

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A simple but efficient filter for transmitting the short-wave ultraviolet light from a mercury arc lamp may be made from two 25 ml. fused silica round-bottomed flasks (Vitreosil T/A4/407). One contains the cobalt-nickel solution ($CoSO_4.7H_2O$ 100 g. + $NiSO_4.7H_2O$ 350 g./l.) and is placed with its centre about 7·5 cm. from the mercury lamp and acts as a lens, forming an image of the lamp about 7·5–8 cm. on the other side. It is stopped down to about 30 mm. diameter by two diaphragms which may be cut in a sheet of metal which is then bent into a U-shape, and the flask pushed in so that the holes grip it. The second flask, which has a glass stopper ground in, is filled with dry chlorine gas. The stopper is greased with vaseline and may with advantage be made hollow so that it can hold some anhydrous calcium chloride held in place with a little glass wool. This flask is so placed that the image of the lamp is formed in its centre, in which position the light passes through virtually undeviated and appears to come from the second flask. The two flasks are connected by a tube of black paper so that no light can emerge without passing through filters. A very powerful and uniform cone of light is produced. Using an 80 W. Mazda MB/V lamp with the glass removed (and choke MRG 508) the area illuminated at a distance of 4 ft. is about 18 × 24 in., the exposure with Kodak reflex document paper and Whatman no. 1 filter paper being about 30 sec.

In making the final adjustment of the positions of the flasks it is important to note that, as the system is not achromatic, the focal length of the flask containing the liquid filter solution is rather shorter at 265 mμ. The field covered by the filter system may, however, be visualized by putting a suitable fluorescent substance such as eosin in ethanol in spots on a sheet of paper and then adjusting the position of the lamp to get maximum evenness of illumination.

The Decarboxylation of Malic Acid by *Lactobacillus arabinosus*

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(*Received* 27 *December* 1950)

Korkes & Ochoa (1948) have shown that *Lactobacillus arabinosus* Strain 17–5 when grown on a medium containing L-malic acid converts L-malic acid into lactic acid and carbon dioxide. D-Malic acid is not attacked, nor does it affect the decarboxylation of the L-isomer. The enzyme system responsible for this reaction was found to include cozymase and manganese ions. The authors state that the enzyme can be used as a specific reagent for the manometric determination of malic acid. In order to use this method it was necessary to collect further information on the general properties of the malic decarboxylase. The present paper reports results of a study of this enzyme system.

Since the completion of this work Ochoa, Salles & Ortiz (1950) and Blanchard, Korkes, del Campillo & Ochoa (1950) have published further information concerning the *Lactobacillus* enzyme and its use in the estimation of L-malic acid.

METHODS

Preparation of cell suspensions. Subcultures of the organism were maintained on a medium containing 5 g. peptone, 20 ml. yeast extract (obtained by boiling baker's yeast with an equal weight of water and filtering), 5 g. glucose and 0·5 g. KH_2PO_4 in 250 ml.; the pH was adjusted to 6·8 with 2 N-NaOH.

The medium used for growing bulk supplies contained, unless otherwise stated, 10 g. Pronutrin (Enzymic casein digest marketed by Herts Pharmaceuticals Ltd., Welwyn Garden City, Herts.), 1 g. Difco Yeast Extract, 10 g. sodium acetate trihydrate, 0·5 g. KH_2PO_4 and 0·5 g. anhydrous $MgSO_4$ in 400 ml.; the initial pH was 7·0. To this were added 50 ml. M-sodium DL-malate solution and 50 ml. 20% glucose. The medium was inoculated with 5–10 ml. of a 24 hr. subculture. After 20 hr. incubation at 38°, the cells were centrifuged, washed successively with 50 and 25 ml. distilled water and finally suspended in 30 ml. water. The yields varied between 2·5 and 3·5 g. wet wt. (up to 650 mg. dry wt.). The suspension was stored in the refrigerator.

Measurement of malic decarboxylase activity. The decarboxylase activity was determined by manometric measurement of the rate of CO_2 evolution. The pH of the solutions used was generally adjusted to 5·0, and in addition 0·025 M (final concentration) acetate buffer, pH 5·0, was present. As a rule, cell suspension, buffer and any other additions except substrates were placed into the main compartment, and the substrate solution into the side arm. The total volume of fluid in the manometer cups was usually 4·0 ml.; the gas space contained air; the temperature of the water bath was 25°, unless otherwise stated.

RESULTS

Effect of yeast extract and glucose. Preliminary experiments showed that addition of glucose and yeast extract increased the activity of malic decarboxylase; their effects were additive (Table 1). Attempts to replace yeast extract by cozymase and a mixture of B vitamins (aneurin, biotin, folic acid, guanine, nicotinamide, *p*-aminobenzoic acid, pantothenic acid, pyridoxine, pimelic acid, riboflavin and xanthine) gave negative results. Among various inorganic substances tested, only Mn^{++} ions and KH_2PO_4 increased the rate of decarboxylation. The effects of Mn^{++}, KH_2PO_4 and glucose were additive (Table 1). In the presence of KH_2PO_4, Mn^{++} and glucose, yeast extract caused no further increase. As yeast extract is known to contain these three substances, its effect is accounted for.

Lag period. When malate was added to a suspension of freshly harvested Lactobacilli there was a lag period of up to 20 min. before the maximum rate of CO_2 evolution was attained (Fig. 1). The lag period was diminished when glucose was added at the same time as malate, and virtually disappeared when the cells were pre-incubated with glucose for 20 min. prior to the addition of malate. The effect of glucose described in the preceding paragraph is not solely due to the abolition of the lag period, because glucose also increases the maximum rate of decarboxylation, especially in thoroughly washed cells. In two typical experiments, the maximum rates of CO_2 evolution without glucose were 25 and 33 μl./5 min., and with glucose, 33 and 48 μl./5 min. respectively. Glucose has thus two effects which may have a common basis. Pre-incubation with KH_2PO_4 and $MnCl_2$ did not affect the lag period.

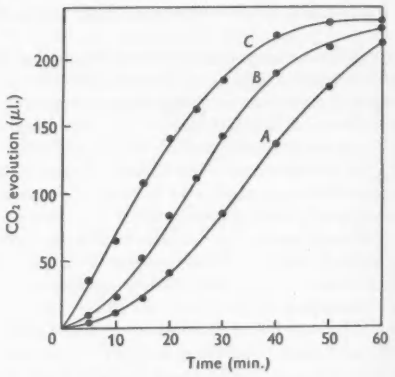

Fig. 1. Effect of glucose on the decarboxylation of malate. 1 mg. dry wt. cells, $2·5 \times 10^{-3}$ M-$MnCl_2$, 5×10^{-2} M-KH_2PO_4, $2·5 \times 10^{-2}$ M-acetate buffer, pH 5·0; 20 μmol. DL-malate. Curve *A*, no glucose; curve *B*, glucose in side arm (with malate); curve *C*, glucose in main compartment (with cells).

Effect of concentration of glucose, manganous chloride, potassium dihydrogen phosphate and buffer. Both the effects of glucose described above were almost independent of the glucose concentration between 5×10^{-2} and 8×10^{-5} M (Table 2); at $1·6 \times 10^{-5}$ M the effects were less marked. The effect of $MnCl_2$ reached a maximum at a concentration of about 2×10^{-3} M, whilst that of KH_2PO_4 increased with concentration up to the surprisingly high value of about 10^{-1} M (Table 3); above this concentration, there was a slight decrease, perhaps because of interference by osmotic forces. The minimum concentra-

Table 1. *Activation of the malic decarboxylase by yeast extract, glucose,* KH_2PO_4 *and* $MnCl_2$

(0·5 ml. freshly harvested cell suspension (10 mg. dry wt.), 5×10^{-2} M-acetate buffer, pH 5·0, 20 μmol. DL-malic acid. The yeast extract was made by placing the carboxylase preparation of Krebs & Johnson (1937) into a boiling-water bath for 5 min. and filtering.)

Exp. no.	Further additions	CO_2 evolved in first 30 min. (μl.)
1	None	39
	0·5 ml. boiled yeast extract	105
	Glucose (5×10^{-3} M)	88
	KH_2PO_4 ($2·5 \times 10^{-2}$ M)	76
	0·5 ml. boiled yeast extract; glucose (5×10^{-3} M)	172
	KH_2PO_4 ($2·5 \times 10^{-2}$ M); glucose (5×10^{-3} M)	179
2	None	8
	Glucose (5×10^{-3} M)	43
	KH_2PO_4 ($2·5 \times 10^{-2}$ M); glucose (5×10^{-3} M)	106
	$MnCl_2$ ($2·5 \times 10^{-4}$ M); glucose (5×10^{-3} M)	160
	KH_2PO_4 ($2·5 \times 10^{-2}$ M); $MnCl_2$ ($2·5 \times 10^{-4}$ M); glucose (5×10^{-3} M)	227

Table 2. *Effect of glucose concentration on the lag period and maximum rate of decarboxylation*

(2 mg. dry wt. cells. $2{\cdot}5 \times 10^{-3}$M-$MnCl_2$, 10^{-1}M-KH_2PO_4, 20 μmol. DL-malate. Substrate in one side arm, all other solutions in main compartment.)

Location of cells	Glucose added (M)	Rate of CO_2 evolution (μl.) over the first four 10 min. intervals, after tipping: Interval 1	Interval 2	Interval 3	Interval 4
Side arm	None	11	31	44	51
	1×10^{-2}	23	60	58	48
	4×10^{-4}	23	61	56	44
	$1{\cdot}6 \times 10^{-5}$	16	49	47	44
Main compartment	1×10^{-2}	65	73	46	35
	4×10^{-4}	64	70	47	41
	$1{\cdot}6 \times 10^{-5}$	43	55	47	48

Table 3. *Effect of dilution on the action of* $MnCl_2$ *and* KH_2PO_4

(1·0 mg. dry wt. cell suspension, $2{\cdot}5 \times 10^{-3}$M-glucose; 20 μmol. DL-malate. 5×10^{-2}M-KH_2PO_4 in Exp. 1; $2{\cdot}5 \times 10^{-3}$M-$MnCl_2$ in Exp. 2.)

Exp. no.	Further addition	CO_2 evolved (μl.): First 15 min.	Second 15 min.
1	None	36	38
	$MnCl_2$ (10^{-2}M)	78	68
	$MnCl_2$ (4×10^{-4}M)	71	63
	$MnCl_2$ ($1{\cdot}6 \times 10^{-5}$M)	54	55
2	None	18	18
	KH_2PO_4 (10^{-1}M)	102	74
	KH_2PO_4 (4×10^{-2}M)	77	75
	KH_2PO_4 ($1{\cdot}6 \times 10^{-4}$M)	30	35
	KH_2PO_4 ($3{\cdot}2 \times 10^{-5}$M)	21	18

Table 4. *Effect of small quantities of glucose,* KH_2PO_4 *and* $MnCl_2$

(1·2 mg. dry wt. cells, 20 μmol. DL-malate. Cell suspension in side bulbs of the cups.)

Glucose (M)	KH_2PO_4 (M)	$MnCl_2$ (M)	CO_2 evolved in first 30 min. (μl.)
None	5×10^{-2}	$2{\cdot}5 \times 10^{-3}$	53
$3{\cdot}2 \times 10^{-6}$	5×10^{-2}	$2{\cdot}5 \times 10^{-3}$	71
$1{\cdot}6 \times 10^{-5}$	5×10^{-2}	$2{\cdot}5 \times 10^{-3}$	86
$2{\cdot}5 \times 10^{-3}$	None	$2{\cdot}5 \times 10^{-3}$	14
$2{\cdot}5 \times 10^{-3}$	$6{\cdot}4 \times 10^{-6}$	$2{\cdot}5 \times 10^{-3}$	16
$2{\cdot}5 \times 10^{-3}$	$3{\cdot}2 \times 10^{-5}$	$2{\cdot}5 \times 10^{-3}$	18
$2{\cdot}5 \times 10^{-3}$	5×10^{-2}	None	31
$2{\cdot}5 \times 10^{-3}$	5×10^{-2}	$3{\cdot}2 \times 10^{-6}$	35
$2{\cdot}5 \times 10^{-3}$	5×10^{-2}	$1{\cdot}6 \times 10^{-5}$	45

tions at which the three activators exert definite effects are shown in Table 4. In this experiment 1 mol. of glucose, $MnCl_2$ and KH_2PO_4 caused additional decarboxylation of 62 (1st half hour), 24 and 9 (1st hr.) mol. of malate respectively. All three substances thus act catalytically.

Increasing concentrations of acetate buffer progressively reduced the rate of decarboxylation. Thus in one experiment the rates with 0·01, 0·025, 0·05 and 0·075M-acetate buffer were 127, 109, 92 and 70 μl./5 min. respectively.

Effects of other substances. As Korkes & Ochoa (1948) have shown that cozymase is required for the decarboxylation of L-malic acid by acetone-powder extracts of Lactobacilli, it was thought that the effect of glucose might be related to the maintenance of the cozymase level in the cells. Experiments were therefore carried out to test whether cozymase could replace glucose. Sufficient $MnCl_2$ and KH_2PO_4 were added to make their concentrations optimal. However, no acceleration by added cozymase (up to 0·25 mg./ml.) was found.

Glucose was found to be replaceable by fructose, mannose, maltose and galactose (in decreasing order of effectiveness), whereas D-arabinose, L-arabinose, D-xylose, D-ribose, sucrose, lactose and glycogen were inactive (Table 5).

Table 5. *Replacement of glucose by other sugars*

(1 and 2 mg. dry wt. cells respectively, for Exps. 1 and 2, 10^{-1}M-KCl, $2{\cdot}5 \times 10^{-3}$M-$MnCl_2$, $2{\cdot}5 \times 10^{-2}$M-acetate buffer, pH 5·0, 30 μmol. DL-malate. Sugars to be tested $2{\cdot}5 \times 10^{-3}$M.)

Exp. no.	Addition	Rate of CO_2 evolution (μl.): First 5 min.	Second 5 min.	Third 5 min.	Fourth 5 min.
1	None	19	43	48	46
	Glucose	59	68	56	49
	Fructose	52	60	54	49
	Galactose	28	48	50	45
	Mannose	55	65	58	49
	Sucrose	18	38	48	51
	Lactose	13	36	48	46
	Maltose	35	52	58	48
	D-Xylose	17	40	46	46
2	None	22	48	52	—
	Glucose	59	58	53	—
	L-Arabinose	15	41	49	—
	D-Arabinose	17	43	48	—
	D-Ribose	25	48	48	—
	Glycogen	19	43	52	—

No acid production (at pH 6·0) was found with sugars which had no effect on the decarboxylation, whilst those which accelerated the decarboxylation were fermented, with the exception of galactose,

which always caused a slight stimulation of decarboxylation, but sometimes failed to show any acid production (Table 6). Fructose was generally fermented at the highest rate, and showed no lag period. The fermentation of glucose showed a lag period of up to 20 min. Mannose was fermented less rapidly than glucose and the lag period exceeded that of glucose. Therefore, there seems to be no quantitative correlation between the rates at which various hexoses are fermented by the cells and the extent to which they accelerate the decarboxylation of malate.

Out of a series of inorganic ions tested only Mg^{++} gave an effect similar to that of Mn^{++} ions. The maximum effect of Mg^{++} was attained at a concentration of 4×10^{-2}M (sixteen times the optimum concentration of Mn^{++}) and was even then less than the effect of Mn^{++} (Table 8). Adenosinetriphosphate did not replace KH_2PO_4, either in the absence or presence of added glucose. Sodium pyrophosphate and freshly prepared sodium metaphosphate were inactive. NaH_2PO_4 also failed to replace KH_2PO_4, indicating that potassium was the active principle of

Table 6. *Comparison between the fermentability of hexoses and their effect on the decarboxylation of malate*

(Decarboxylation and fermentation were measured in solutions containing $2{\cdot}5 \times 10^{-3}$M-$MnCl_2$ and 10^{-1}M-KCl; for decarboxylation, $2{\cdot}5 \times 10^{-2}$M-acetate buffer, pH 5·0, and 40 μmol. DL-malate were also present; for fermentation, the solution also contained $1{\cdot}32 \times 10^{-2}$M-bicarbonate in a gas phase of 100% CO_2; this, at 30°, gives a pH of 6·0. Sugars were added to a final concentration of 5×10^{-3}M. Decarboxylation and acid production were measured simultaneously using the same cell suspension.)

Exp. no.	Sugar added	Amount of cells (mg. dry wt.)	Lag period before reaching maximum rate of acid production (min.)	Acid production (μl./hr.)	Maximum acceleration of decarboxylation (% increase over the rate without added sugar)
1	Glucose	1·4	20	243	140
	Fructose	1·4	0–5	289	72
	Mannose	1·4	30	39	75
	Galactose	1·4	—	0	39
2	Glucose	2·1	5	367	53
	Fructose	2·1	5	386	49
	Galactose	2·1	—	0	29
3	Glucose	2·3	10	320	85
	Mannose	2·3	20	287	85
	Galactose	2·3	—	32	37

Lactate was found inactive, but pyruvate accelerated the decarboxylation (Table 7), although much less markedly than equivalent concentrations of glucose. Pyruvate, in contrast to glucose, had no effect at concentrations below $2{\cdot}5 \times 10^{-4}$M. Above a concentration of 5×10^{-3}M, pyruvate itself was found to undergo decarboxylation after an initial

Table 7. *Replacement of glucose by pyruvate*

(1·9 mg. dry wt. cells, $2{\cdot}5 \times 10^{-3}$M-$MnCl_2$, 5×10^{-2}M-KH_2PO_4, 20 μmol. DL-malate.)

Further additions	CO_2 evolved in first 15 min. (μl.)
None	67
Glucose ($2{\cdot}5 \times 10^{-3}$M)	137
Pyruvate ($2{\cdot}5 \times 10^{-2}$M)	121
Pyruvate ($2{\cdot}5 \times 10^{-3}$M)	102
Pyruvate ($2{\cdot}5 \times 10^{-5}$M)	65

lag period, a reaction which will be described in a separate communication. The increased CO_2 output observed when both malate and pyruvate were present was not due to decomposition of pyruvate but to an acceleration of the decarboxylation of malate.

KH_2PO_4. Table 9 shows that KCl had in fact a similar effect and that, in the presence of KCl, NaH_2PO_4 was still inactive. KCl was slightly more

Table 8. *Comparison of the effectiveness of* Mn^{++} *and* Mg^{++}

(4 mg. dry wt. cells, $2{\cdot}5 \times 10^{-3}$M-glucose, 10^{-1}M-KCl, 40 μmol. DL-malate.)

Metal ion added	Final concn. (M)	Increase in activity (compared to the rate without added metal) (%)
Mn^{++}	$2{\cdot}5 \times 10^{-3}$	100
Mg^{++}	$2{\cdot}5 \times 10^{-3}$	57
Mg^{++}	1×10^{-2}	68
Mg^{++}	4×10^{-2}	70

effective than equivalent amounts of KH_2PO_4 between concentrations of 10^{-1} and $3{\cdot}2 \times 10^{-5}$M. Since NaCl was inactive, it may be concluded that the observed acceleration of malate decarboxylation by KH_2PO_4 is entirely due to potassium. Amounts of sodium ions, added as NaCl, up to 40 times the excess of potassium present, did not reduce the activity appreciably, and 0·1 M-Li^+ and NH_4^+ failed to

Table 9. *Effect of* KCl, *and failure of* Na_2HPO_4 *to increase activity*

(2·4 mg. dry wt. cells, $2·5 \times 10^{-3}$M-$MnCl_2$, $2·5 \times 10^{-3}$M-glucose, 40 μmol. DL-malic acid. The Na_2HPO_4 used was adjusted to pH 5.)

Further addition	CO_2 evolved in first 10 min. (μl.)
None	24
Sodium phosphate ($2·5 \times 10^{-3}$M)	24
Sodium phosphate ($2·5 \times 10^{-3}$M); KCl ($1·7 \times 10^{-1}$M)	82
Sodium phosphate ($2·5 \times 10^{-3}$M); KCl ($1·7 \times 10^{-2}$M)	92
Sodium phosphate ($2·5 \times 10^{-3}$M); KCl ($1·7 \times 10^{-3}$M)	70
Sodium phosphate ($2·5 \times 10^{-3}$M); KCl ($1·7 \times 10^{-4}$M)	58
KCl ($1·7 \times 10^{-2}$M)	96
KCl ($1·7 \times 10^{-2}$M); sodium phosphate (5×10^{-2}M)	92
KCl ($1·7 \times 10^{-2}$M); sodium phosphate (5×10^{-3}M)	101
KCl ($1·7 \times 10^{-2}$M); sodium phosphate (5×10^{-4}M)	92
KCl ($1·7 \times 10^{-2}$M); sodium phosphate (5×10^{-5}M)	93

Table 10. *Replacement of potassium by rubidium and caesium*

(2 mg. dry wt. cells, $2·5 \times 10^{-3}$M-glucose, $2·5 \times 10^{-3}$M-$MnCl_2$, 40 μmol. DL-malate.)

Exp. no.	Additions	Maximum rate of CO_2 evolution (μl./5 min.)
1	None	20
	KCl ($2·5 \times 10^{-2}$M)	64
	KCl ($1·6 \times 10^{-4}$M)	64
	RbCl ($2·5 \times 10^{-2}$M)	64
	RbCl ($1·6 \times 10^{-4}$M)	67
	CsCl ($2·5 \times 10^{-2}$M)	54
	CsCl ($1·6 \times 10^{-4}$M)	35
2	None	31
	KCl ($2·5 \times 10^{-2}$M)	71
	RbCl ($2·5 \times 10^{-2}$M)	76
	CsCl ($2·5 \times 10^{-2}$M)	62

affect the rate of decarboxylation in the presence of the same concentration of KCl. Potassium could be replaced entirely by rubidium, which was as active at all concentrations tested (Table 10). Caesium was somewhat less effective. The sample of CsCl used was of the 'Analar' quality of British Drug Houses Ltd.; no examination of its potassium and rubidium content was carried out.

The effect of anions. Amongst various salts of potassium tested, the chloride and phosphate were the most effective. Sulphate, nitrate and acetate inhibited at higher concentrations (Table 11).

Comparison of the decarboxylation in suspensions of intact and impaired cells. Aqueous suspensions of acetone powders (prepared by slowly pouring a concentrated suspension of cells into 10 times its volume

Table 11. *Comparison of the effect of* KCl, KNO_3, K_2SO_4 *and* CH_3COOK

(1·4 mg. dry wt. cells, $2·5 \times 10^{-3}$M-glucose, $2·5 \times 10^{-3}$M-$MnCl_2$, 20 μmol. DL-malate. Part *A*: 10^{-2}M-potassium acetate buffer, pH 5·0. Part *B*: $2·5 \times 10^{-2}$M-sodium acetate buffer, pH 5·0.)

Part	Additions	CO_2 evolved in first 30 min. (μl.)
A	None	41
	KCl ($7·5 \times 10^{-2}$M)	130
	KCl ($1·5 \times 10^{-1}$M)	147
	KCl (3×10^{-1}M)	151
	K_2SO_4 ($7·5 \times 10^{-2}$M)	79
	K_2SO_4 ($1·5 \times 10^{-1}$M)	82
	K_2SO_4 (3×10^{-1}M)	97
	KNO_3 ($7·5 \times 10^{-2}$M)	89
	KNO_3 ($1·5 \times 10^{-1}$M)	77
	KNO_3 (3×10^{-1}M)	66
B	None	29
	CH_3COOK (5×10^{-2}M), pH 5·0	152
	CH_3COOK (5×10^{-2}M), pH 5·0	121
	CH_3COOK (10^{-1}M), pH 5·0	106

Table 12. *Constituents of the enzyme system in freeze-dried cells*

(0·2 ml. (2 mg. dry wt.) suspension of freeze-dried cells, 4×10^{-2}M-acetate buffer, pH 5·8, 30 μmol. DL-malate. The complete system further contained 5×10^{-3}M-glucose or pyruvate, 5×10^{-3}M-$MnCl_2$, 5×10^{-2}M-KCl and 0·2 mg. cozymase.)

Omissions or substitutions	CO_2 evolved in 1 hr. (μl.)
Complete system (with glucose)	184
Complete system (with pyruvate)	189
Complete system (with glucose and pyruvate)	179
Complete system (substituting KH_2PO_4 for KCl)	161
No glycose or pyruvate	146
No cozymase	43
No $MnCl_2$	70
No KCl	7

Table 13. *The effect of* pH *and buffer composition on enzymic activity*

(0·2 ml. enzyme suspension (2 mg. dry wt.), $2·5 \times 10^{-3}$ M-glucose, $2·5 \times 10^{-3}$ M-$MnCl_2$, 10^{-1} M-KCl, 40 μmol. DL-malate. 0·2 mg. cozymase in Exp. 3. Buffer concentration, 5×10^{-2} M throughout.)

Exp. no.	System	pH of buffer	Composition of buffer	pH at conclusion of the experiment (glass electrode)	Location of enzyme	CO_2 evolved (μl.) First 10 min.	Second 10 min.	Third 10 min.
1	Intact cells	3·6	Acetate	4·0	Main compartment	45	58	59
		4·0	Acetate	4·2	Main compartment	70	102	85
		4·5	Acetate	4·6	Main compartment	111	113	83
		4·7	Acetate	4·7	Main compartment	112	105	80
		5·0	Acetate	4·9	Main compartment	129	109	81
		5·4	Acetate	5·4	Main compartment	77	74	58
2	Intact cells	3·6	Acetate	4·0	Side arm	54	84	98
		4·0	Acetate	4·2	Side arm	61	111	101
		4·5	Acetate	4·6	Side arm	62	109	96
		4·7	Acetate	4·7	Side arm	53	106	86
		5·0	Acetate	4·9	Side arm	47	109	89
		5·4	Acetate	5·4	Side arm	28	67	57
3	Suspension of freeze-dried cells	4·7	Acetate	4·7	Main compartment	15	13	11
		5·0	Acetate	4·9	Main compartment	23	19	20
		5·4	Acetate	5·5	Main compartment	54	46	41
		5·8	Acetate	5·7	Main compartment	60	48	44
		6·1	Acetate	5·9	Main compartment	61	46	44
		5·8	Phosphate	5·8	Main compartment	57	43	42
		6·1	Phosphate	6·0	Main compartment	55	45	40
		6·5	Phosphate	6·4	Main compartment	45	36	35
		6·9	Phosphate	6·8	Main compartment	23	22	20
							First 15 min.	
4	Intact cells	4·9	KH_2PO_4 only	—	Main compartment		205	
		5·0	Acetate	—	Main compartment		175	
		5·0	Citrate	—	Main compartment		183	
		5·1	Phthalate	—	Main compartment		68	

of ice-cold acetone) were inactive, even when 0·5 mg. cozymase as well as glucose, $MnCl_2$ and KH_2PO_4 were added. Aqueous suspensions of cells frozen at $-12°$ and then dried *in vacuo* over P_2O_5 showed up to half the activity of the original intact cells in the presence of Mn^{++}, K^+ (KH_2PO_4 could still be replaced by KCl), glucose (or pyruvate) and cozymase (Table 12). Omission of glucose or pyruvate from this system caused much less reduction in activity than omission of cozymase.

After extraction up to 14 hr. of freeze-dried cells at 25° with sufficient water to give the same cell concentration as the original suspension, 90 % of the activity still remained in the non-soluble residue, and 10 % was in the supernatant. Extraction at higher temperatures caused inactivation.

Intact and freeze-dried cells differed in respect to their pH optima of malate decarboxylation and their activities in various buffers (Table 13). The pH optimum of the enzyme system in intact cells was 4·5–5·0. Suspensions of freeze-dried cells showed a pH optimum around 6·0. Intact cells were more active in phosphate than in acetate buffer, whereas freeze-dried cells were at least as active in acetate buffer as in phosphate buffer of the same pH.

The half maximum rate of decarboxylation was attained at a substrate concentration of $1·8 \times 10^{-3}$ M in intact cells, and 8×10^{-3} M in freeze-dried cells.

The enzyme in suspensions of intact cells had a temperature optimum at 35°; with freeze-dried cells the highest initial rates of CO_2 evolution were observed at 40°, but at that temperature there was subsequently a marked inactivation.

DISCUSSION

The main new finding is the accelerating effect of potassium ions on the rate of decarboxylation of malate. Potassium ions are already known to be required for several other enzymic processes, namely the phosphate transfer between pyruvate and adenosinetriphosphate (Lardy & Ziegler, 1945), the oxidative decarboxylation of malate to pyruvate by *Moraxella lwoffi* (Lwoff & Ionesco, 1947) and possibly the conversion of hexosemonophosphate to hexosediphosphate by yeast extracts (Munz, 1947). Two of these are thus directly concerned with phos-

phorylation, and the third, the oxidative decarboxylation, is assumed to involve phosphorylation.

The conversion of L-malic acid into pyruvic acid in *Moraxella* resembles the reaction investigated in this paper in that both involve oxido-reduction; the two reactions probably have some stages in common, but in the case of *Moraxella* molecular oxygen takes part and the product is pyruvate, whereas in *Lactobacillus* the oxido-reduction is internally compensated and the product is lactate. In *Moraxella*, both rubidium and caesium replace potassium; in *Lactobacillus*, rubidium is as effective as potassium; the effect of caesium was less pronounced; although the sample of caesium used was of 'Analar' quality, the possibility cannot be excluded that the observed acceleration of the decarboxylation of malate was due to contamination by potassium.

The main differences between the decarboxylation of malate by intact cells of *Lactobacillus* and by the acetone powder extracts of Korkes & Ochoa (1948), concern the effects of glucose and cozymase. In the intact cells glucose catalytically accelerates decarboxylation whilst added cozymase is inactive; in acetone-powder extracts (which are unlikely to metabolize glucose under the experimental conditions used) glucose has no effect, but added cozymase is required for full activity. The independence of the enzyme in intact cells of added cozymase is presumably due to the fact that they already contain optimal amounts of the coenzyme (Hughes, 1950). The detailed mechanism of the glucose effect remains obscure: there is a qualitative correlation between the fermentability of sugars and their ability to accelerate the decarboxylation of malate. It is perhaps a linked oxido-reduction involving malate and a derivative of glucose; since pyruvate has been shown to accelerate the decarboxylation of malate, the effect of glucose may to some extent be explained by assuming that glucose gives rise to pyruvate; however, the effect of glucose was always more marked than that of pyruvate, and therefore other derivatives may well be concerned in this linked reaction.

SUMMARY

1. The decarboxylation of L-malic acid by washed suspensions of malate-adapted cells of *Lactobacillus arabinosus* is accelerated by glucose and yeast extract, the effects being additive.

2. Yeast extract can be replaced entirely by manganous chloride and potassium dihydrogen phosphate. The action of potassium dihydrogen phosphate is wholly due to potassium. The latter can be replaced by rubidium, and possibly by caesium, but not by lithium, sodium or ammonium.

3. The effectiveness of glucose is increased by pre-incubating it with the cells, and is equal between concentrations of 10^{-4} and 10^{-1}M. The optimum concentrations of manganous chloride and potassium dihydrogen phosphate are 10^{-3} and 10^{-1}M respectively, but all three substances act catalytically.

4. Fructose, mannose, galactose and maltose are, like glucose, fermented and increase the rate of decarboxylation. Unfermentable substances do not. There is no quantitative correlation between the fermentability of hexoses and their effect on decarboxylation.

5. Pyruvate accelerates decarboxylation of malate, its effect being smaller than that of glucose. At higher concentrations (above 5×10^{-3}M final concentration) pyruvate itself undergoes decarboxylation.

6. Suspensions of freeze-dried cells show up to half the activity of the original whole cells. They require cozymase, are affected only slightly by glucose, and differ from suspensions of intact cells in their pH and temperature optima, and their substrate-activity curve.

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A Study of the Polyphenols in Tea Leaf by Paper Chromatography

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Earlier work on the isolation and characterization of the components of the so-called tea tannin, reviewed by Harrison & Roberts (1939), relied mainly on the classical methods of selective extraction and precipitation. The development of partition chromatography as a preparative method has enabled much further progress to be made in the elucidation of the nature of these polyphenols. Bradfield and coworkers have isolated seven catechins in Ceylon green tea, namely L-gallocatechin, DL-gallocatechin, L-*epi*catechin, catechin, L-gallocatechin gallate, L-*epi*catechin gallate and a galloyl ester of gallocatechin (Bradfield, 1946; Bradfield, Penney & Wright, 1947; Bradfield & Penney, 1948). Later, Bradfield & Bate-Smith (1950) suggested that L-gallocatechin and L-gallocatechin gallate might more accurately be identified as L-*epi*gallocatechin and L-*epi*gallocatechin gallate respectively; whilst the galloyl ester of gallocatechin, i.e. the 'substance 2*a*' of Bradfield & Penney (1948), might be the galloyl ester of L-gallocatechin. Further evidence in support and extension of these views will be considered in this communication.

The success of preparative partition chromatography suggested that useful information might be obtained by applying the methods of paper chromatography (Consden, Gordon & Martin, 1944) to a study of the catechins and other polyphenols in tea, and this communication describes the provisional identification of the main polyphenols in tea leaf. The effect of high temperatures during extraction is found not only to affect the relative proportions of some of the various polyphenols but may also result in the production of artifacts, not necessarily present in the natural state. The behaviour of individual polyphenols on enzymic oxidation is discussed. The presence of a large number of so far unidentified anthoxanthins is recorded.

METHODS AND MATERIALS

Sources of polyphenols

Fresh juice. A fine mince of plucked tea shoots is squeezed by hand through fine cloth. The resultant juice is clarified by centrifuging for 5 min., rapidly heated to 100° to prevent enzymic oxidation, and then cooled to room temperature.

Reconstituted juice. It is not always convenient to examine leaf at the time of plucking, and it has been found that identical chromatograms are obtained if the leaf is dried in a current of air heated to 90°, powdered, and subsequently ground with three times its weight of water. The 'reconstituted' juice is then obtained by squeezing through cloth and centrifuging as above. In the case of manufactured tea the product is powdered and mixed with twice its weight of water, after which the same procedure is followed.

Catechin preparations. Mixed tea catechins obtained by the method described by Roberts & Wood (1950) are referred to as precipitated catechins. Other catechin preparations have been obtained essentially by the method described by Bradfield *et al.* (1947). The final product, referred to subsequently as amorphous tea catechin, is obtained by removing the ethyl acetate *in vacuo* in a current of CO_2, taking up the product in the minimum of acetone, and evaporating to dryness.

Chromatographic technique

Apparatus. For two-dimensional analysis cabinets similar to that described by Dent (1948) are used, the one for *n*-butanol-acetic acid being lined with aluminium sheet. In both cabinets the solvent trough is of stainless steel. Sheets of Whatman no. 1 paper have been used throughout.

Quantities required for chromatography. 35 μl. Juice (fresh or reconstituted) represents a suitable quantity for analysis. This quantity is measured out in two portions, allowing the first portion to dry before adding the second. With catechin preparations 20 μl. of a solution containing 100 mg./ml. is a suitable amount.

Solvents. Phenol, saturated with water, is used as the first solvent. For the second solvent the non-aqueous phase of a mixture of *n*-butanol (40%), acetic acid (10%), and water (50%) was first employed, but temperature fluctuations often resulted in separation into two phases. This was avoided by making up a mixture of *n*-butanol (80%) and acetic acid (20%), and adding water just short of the amount required for saturation. Percentages are v/v. A reproducible pattern of spots, and no separation of the solvent into two phases, has resulted.

Spray fluids. For general use $FeCl_3$ (0·1%, w/v) in water or $FeNH_4(SO_4)_2.12H_2O$ (0·2%, w/v) have been found of most value, since they differentiate between catechol and pyrogallol groupings, the former giving a green colour, the latter a dark grey-blue. When both groupings are present in the same molecule, e.g. L-*epi*catechin gallate, the colour is also dark grey-blue. The spots do not always develop their full intensity on spraying, particularly if the paper still contains traces of acetic acid. By holding an opened bottle of ammonia close to each spot full intensities are developed. The spots for catechin and *epi*catechin, if subjected to this treatment, turn dark blue and are then indistinguishable from the other catechins. Some of the anthoxanthins give characteristic, although much weaker, colours with ferric salts. KCN (1%, w/v) has been used to detect gallic acid,

giving an immediate pink colour which fades rapidly. Catechin gallates give an orange-pink which fades, and then gives place to various shades of buff, brown and orange. To detect anthoxanthins the dry chromatogram (before spraying) is exposed to ammonia vapour; the anthoxanthins give temporary yellow colours.

R_F values. R_F values vary considerably with temperature, and it is impracticable to maintain a constant temperature in our laboratory throughout the year. Individual catechins, however, are readily recognized by their position in the overall pattern of spots. R_F values as such are not measured, but they are roughly indicated in Fig. 1, representing average conditions (25°).

RESULTS

Chromatograms of fresh juice and of catechin preparations

A typical two-way chromatogram of fresh juice is illustrated in Fig. 1, the size of the ovals indicating the relative intensities of the spots. In this particular case spraying with $FeCl_3$ reveals nine components, two of which, 9 and 10, give green spots, while the remainder give dark blue-grey spots. After a few days spots 2 and 3 fade to a brown colour.

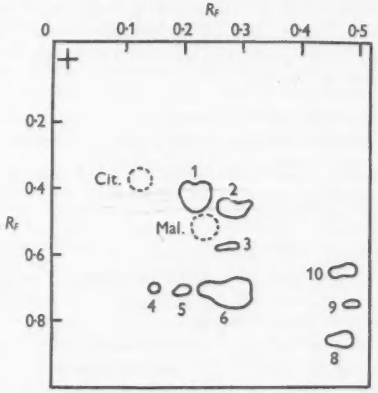

Fig. 1. Chromatogram of tea-leaf juice, showing positions of major polyphenolic components (spots 1–6 and 8–10), and citric and malic acids. The chromatogram was run first from left to right with phenol as solvent, and then downwards with *n*-butanol-acetic acid. R_F values are approximate only. Colour reagent $FeCl_3$. Size of spot roughly proportional to colour intensity.

The fading of spot 2 is of particular value, owing to the frequent merging of spots 1 and 2 in chromatograms. In such cases only part of the spot fades to brown, so that evidence for both components is available when separation is poor.

The chromatogram (Fig. 2) for precipitated catechins, prepared from the same batch of leaf, is not dissimilar save that spot 1 is absent, a new spot (no. 7) appears and the relative intensities of some of the other spots are affected. The relative proportions of the ten components in precipitated and amorphous preparations and in fresh juice are indicated in Table 1.

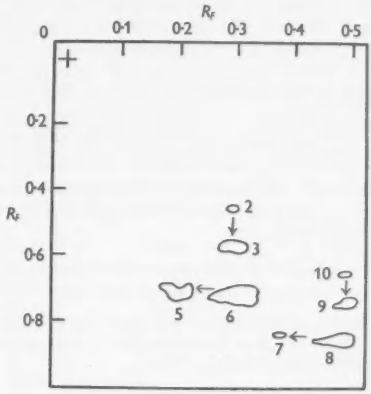

Fig. 2. Chromatogram of amorphous or precipitated catechin preparation. The arrows indicate the direction of epimeric changes caused by heating the solutions. Conditions of chromatography as in Fig. 1.

The fact that spot 1 is either faint or absent altogether in chromatograms of catechin preparations indicates that this component is not extractable from aqueous solution by ethyl acetate. Spots 5 and 8 are relatively more intense in catechin preparations

Table 1. *Comparison of fresh juice with crude catechin preparations*

(Relative colour intensities after chromatography and colour development with $FeCl_3$. Spot numbers refer to Figs. 1 and 2 and colour intensity is indicated by the scale + + + + very strong to (+) trace.)

Spot no.	Fresh juice	Amorphous catechin	Precipitated catechin
1	+ + +	(+)	.
2	(+)	(+)	.
3	+(+)	+(+)	+(+)
4	+	+	+
5	+(+)	+ +(+)	+ + +(+)
6	+ + +(+)	+ + + +	+ + + +
7	.	(+)	+
8	+(+)	+ +(+)	+ +(+)
9	+	+(+)	+(+)
10	+	+	+

than in the original juice, suggesting that selective extraction and precipitation may be operative. The appearance of the new spot 7 suggests that the treatment given during preparation may result in the production of substances not present in the original juice.

In the preparation of precipitated catechins the green leaf is refluxed for 1 hr., and in the procedure of Bradfield *et al.* (1947), which forms the first stage of our method of preparing amorphous catechins, there are several stages involving refluxing or boiling. These preparations therefore differ from fresh juice in that they have been maintained at a relatively high temperature for an appreciable period, and not for the short time required for inactivation of enzymes in the fresh juice. To test the effect of such high temperatures chromatograms were run with fresh juice and fresh juice refluxed for 1 hr. Typical results of such an experiment are shown in Table 2. Spots 2 and 6 show a definite decrease, spots 3 and 9 show an increase in intensity and spots 5 and 7 make their appearance.

Table 2. *Effect of heat treatment on the chromatographic behaviour of fresh juice*

(The spot numbers refer to the spots indicated in Figs. 1 and 2. Relative colour intensities ($FeCl_3$) are indicated in the same way as in Table 1.)

Spot no.	Juice unboiled	Juice boiled	Juice from untreated mince	Juice from autoclaved mince
1	+ +	+ +	+ + +	+ +(+)
2	+ +(+)	+(+)	+ +(+)	+(+)
3	+(+)	+ +(+)	(+)	+(+)
4	(+)	+	+	+
5	.	+(+)	.	+(+)
6	+ + +(+)	+ + +	+ + + +	+ + +
7	.	(+)	.	(+)
8	+	+	+ +	+
9	(+)	+	(+)	+
10	+(+)	+(+)	+(+)	+

It is not possible to express slight variations adequately by the method of estimation adopted, and in this particular case there is no apparent change in the intensity of spots 8 and 10. In other experiments decreases in one or both of these spots have been observed (cf. also Table 3). In such cases, however, changes in some of the other components have been less definite. Greater effects are observed after autoclaving the mince at 130° before expressing the juice, and as a result of such treatment significant decreases are observed in the intensities of spots 2, 6, 8 and 10. Spots 3 and 9 show increased intensity, whilst spots 5 and 7 appear on the chromatogram as a result of the autoclaving treatment (Table 2).

Epimerization

During the earlier stages of this work the only catechin available was Merck's catechin. Addition of this substance to tea-leaf juice intensified spot 9 in the resultant chromatogram. It seemed reasonable to deduce that spot 10, which like 9 also gives a green colour on spraying with $FeCl_3$, was due to L-*epi*catechin. This was confirmed later by a comparison of R_F values in *n*-butanol-acetic acid with those obtained by Bradfield & Bate-Smith (1950), and by addition of authentic specimens of L-*epi*catechin to the expressed juice, which resulted in intensification of spot 10.

The changes in the relative intensities of spots 9 and 10 as a result of boiling appear, therefore, to be due to epimerization, L-*epi*catechin being converted into catechin. Confirmation was provided by refluxing a solution of catechin for 2 hr.; this treatment resulted in the appearance of a second green spot, in the position expected for L-*epi*catechin, on a one-way chromatogram run with *n*-butanol-acetic acid and sprayed with $FeCl_3$. This also indicates that the epimerization is reversible.

The possibility therefore arises that spots 2, 3 and 5–8 may represent three further pairs of epimers. Grouping into pairs was facilitated by the observation that some sources of leaf were lacking in certain catechins. On one occasion juice expressed from the leaf of one clone of tea (14/5/18) was found to contain no catechins corresponding with spots 2 and 3. Leaf was also available from an unidentified *Camellia* from northern Burma, which although botanically distinct, bears many resemblances to tea, and can be crossed with it. The juice expressed from leaves from this source gave a chromatogram consisting of spots 1, 8 and 10 together with others corresponding to hitherto unidentified polyphenols.

The effects of refluxing juice from these two sources are given in Table 3.

Table 3. *The effect of boiling juice lacking in certain catechins on its chromatographic behaviour*

(The spot numbers refer to Figs. 1 and 2. Colour intensity after spraying with $FeCl_3$ is indicated by the same convention as in Table 1.)

Spot no.	Clone 14/5/18		*Camellia* sp.	
	Unboiled	Boiled	Unboiled	Boiled
1	+ + +	+ + +	+ + + +	+ + + +
2	.	.	.	.
3	.	.	.	.
4	.	.	.	.
5	.	+(+)	.	.
6	+ + + +	+ + +	.	.
7	.	(+)	.	(+)
8	+(+)	+	+ +	+
9	(+)	(+)	.	(+)
10	+	(+)	+	(+)

From the results in Tables 2 and 3 it will be seen that spot 3 only increases in intensity, as a result of boiling, if spot 2 is originally present. Further evidence for the pairing of spots 2 and 3 is their similar behaviour in fading to a brown colour after a few days. An increase in the intensity of spot 5 (or its appearance) only takes place if the catechin corresponding to spot 6 is present in the unboiled juice. Finally, the appearance of spot 7, after boiling a juice containing catechins corresponding to spots 8 and 10, indicates that spots 7 and 8 form the

third pair, as spot 10 has already been shown to be paired with spot 9.

The relationships between the four pairs of catechins are shown diagrammatically in Fig. 2; an arrow linking each pair indicates the direction of the change taking place on boiling. It is noteworthy that each pair of catechins is represented by spots very close to each other on the chromatogram, which is consistent with the differences in chemical composition being in each case relatively small.

Identification of spots on the chromatogram

In view of the isolation of seven catechins in tea leaf (Bradfield *et al.* 1947; Bradfield & Penney, 1948) it appeared likely that these catechins were responsible for seven of the ten spots under consideration. As Bradfield (1946) found that the substance originally described as L-gallocatechin gallate was always the most abundant, it seemed that it might be spot 6 on our chromatograms.

If juice is inoculated with the spores of *Aspergillus niger* a vigorous growth of the mould develops on the surface of the juice. According to Deijs & Dijkman (1936). *A. niger* contains a powerful tannase capable of eliminating gallic acid from such galloyl esters as are found in tea, so that one would expect a marked decrease in the content of galloyl esters and a corresponding increase in gallic acid, all of which changes should be detectable on the chromatogram. Autoclaved juice is more suitable for such an experiment than fresh juice as it contains appreciable amounts of the polyphenols corresponding with spots 5 and 7.

In one experiment on autoclaved juice suitable growth of the mould was attained 3 days after inoculation. A chromatogram of the inoculated juice was then run and compared with that of the control (Table 4). The result of such treatment was a marked decrease in the strength of spots 5–8 and a considerable increase in that of spot 4, which latter is thus indicated as gallic acid. It follows that spots 5–8 are probably galloyl esters. As spot 6 has already been provisionally identified, spot 8 probably corresponds with L-*epi*catechin gallate, a known component of the tea leaf, while spots 5 and 7 normally absent from unboiled tea juice are probably galloyl esters formed from 6 and 8 respectively, on boiling. This leaves spots 2 and 3 unidentified, and by elimination these may be L-*epi*gallocatechin and DL-gallocatechin respectively. The *epi* structure is allotted to the catechin of lower R_F in *n*-butanol-acetic acid by analogy with the identification of L-*epi*catechin with spot 10.

Subsequent to these preliminary identifications, authentic specimens of the following catechins were received: L-*epi*gallocatechin, DL-gallocatechin, L-*epi*gallocatechin gallate, gallocatechin-a gallate, L-*epi*catechin gallate and L-*epi*catechin. The nomenclature is that as revised by Bradfield & Bate-Smith (1950).

By addition of suitable amounts of these substances to tea juice, intensifications of the appropriate spots occurred, enabling more positive identifications to be made. In some cases where the original spot is very strong, juice from fermented tea proved more suitable, as on fermentation several of the spots decrease very considerably in intensity.

Table 4. *Effect of incubation of juice with* Aspergillus niger *on its chromatographic behaviour*

(Spot numbers as in Figs. 1 and 2. Colour intensity after spraying with $FeCl_3$ as in Table 1.)

Spot no.	Control	Incubated with *A. niger*
1	+ + +	+ + + +
2	+	+(+)
3	+ +	+ +
4	+ +	+ + + +
5	+ + +	+(+)
6	+ + +	+
7	+	(+)
8	+ +	(+)
9	+(+)	+(+)
10	+(+)	+(+)

These experiments support the provisional identifications of spots 2, 3, 6 and 8, and identify spot 5 with gallocatechin-a gallate. Provisional identifications of spots 9 and 10 have already been made. The possible identification of spot 7 as catechin-a gallate is treated in the discussion.

The provisional identification of spot 4 with gallic acid follows from the results of inoculation with *Aspergillus niger*. Its acidic nature is indicated by the results of spraying with bromocresol green (Lugg & Overell, 1947) while spraying with potassium cyanide gives the characteristic fading pink colour reaction for gallic acid. Confirmation is afforded by adding gallic acid to juice when the resultant chromatogram has spot 4 greatly intensified.

The identity of spot 1 has not yet been established, but the following considerations lead to its tentative identification with *m*-digallic acid. Spraying the chromatogram with bromocresol green gives two intensely yellow spots corresponding with spots 1 and 4 (other organic acids are also shown up). Subsequent spraying with ferric chloride changes these two yellow spots to a dark blue-grey. Spot 1, therefore, is an organic acid containing a pyrogallol group.

An aqueous solution containing no other polyphenols apart from traces of gallic acid can be obtained by exhaustive extraction of a green leaf infusion with ethyl acetate. The aqueous layer on concentration and chromatography has a strong spot 1 and also contains a little gallic acid. This solution on hydrolysis with hydrochloric acid gives gallic acid as the only polyphenolic product as

judged by paper chromatography of the hydrolysate. No characteristic dark-red precipitate is formed, such as is obtained when catechins are similarly treated. A more concentrated solution was obtained by precipitation with lead acetate, decomposition of the lead salt with N-sulphuric acid, and subsequent concentration. This solution gave no pine-shaving test for phloroglucinol, and no precipitate with bromine water. On the other hand, it gave a precipitate with gelatin. Judging by its persistence in fermented tea as discussed later, it is oxidized only slowly by tea oxidase.

The fact that spot 1 is an acid, and gives gallic acid on hydrolysis, suggests its possible identity with *m*-digallic acid. This is in accordance with the results of the tests with the pine shaving, bromine water and gelatin, and its failure to be extracted from aqueous solution by ethyl acetate. Further work in connexion with the identification of this polyphenolic acid is in progress.

Provisional identifications of the ten most frequently occurring spots are given in Table 5.

Table 5. *Provisional identification of polyphenols on chromatograms*

Spot no.	Polyphenol
1	*m*-Digallic acid (?)
2	L-*epi*Gallocatechin
3	DL-Gallocatechin
4	Gallic acid
5	Gallocatechin-a gallate
6	L-*epi*Gallocatechin gallate
7	Catechin-a gallate (?)
8	L-*epi*Catechin gallate
9	Catechin
10	L-*epi*Catechin

Other substances appearing on the chromatograms

Polyphenols. The ten components discussed in detail above appear to be those which most generally occur in teas grown in north-east India. In addition, however, other spots, the positions of which are given in Fig. 3, giving dark blue-grey colours with ferric chloride are observed from time to time. Normally they occur in traces only, although towards the end of the year spot 14 is sometimes present as a major component. So far no progress has been made in their identification.

Anthoxanthins. Any spot which gives a temporary yellow colour with ammonia vapour, and subsequently a faint green, buff or olive colour on spraying with ferric chloride, is referred to as an anthoxanthin. As many as fourteen anthoxanthins have been differentiated on one chromatogram. The more commonly occurring spots are shown in Fig. 4; on spraying with ferric chloride spots *A* 6, *A* 7 and *A* 8 give green colours while the remainder give buff or brownish olive colours. Spots *A* 1 and *A* 2 appear to be characteristic of a particular botanical type, as they occur only in bushes of China type or in bushes which have originated from such a type by hybridization. So far little progress has been made in the identification of the anthoxanthins. Judging by the R_F values in *n*-butanol-acetic acid given by Bate-Smith & Westall (1950) no spot corresponding to kaempferol is present on any of our chromatograms, although Oshima & Ka (1936) claim to have established its presence in tea. Quercitrin has been added to tea juice and travels to a position (*A* 11) not normally occupied by any natural anthoxanthin, although some sources show a faint spot in this position. It does not therefore appear to be of general occurrence in north-east Indian teas, although its presence has been reported by Deuss (1923). Spot *A* 4 has been identified as rutin.

Organic acids. After spraying with ferric chloride the paper on drying is a very faint brown in colour, due presumably to deposition of ferric hydroxide. There are, however, a number of clear patches where the paper retains its original white colour. If the chromatogram is first sprayed with bromocresol green these clear patches show up as yellow spots on a blue to green background. The clear patches may therefore be ascribed to free acids which presumably prevent precipitation of ferric hydroxide. Identification of all such patches has not yet been successful, but two of them correspond to citric and malic acids and are marked as Cit. and Mal. in Fig. 1.

Variation in chemical composition between individual bushes

Owing to the extensive hybridization that has occurred in the past it is difficult to obtain even a small plot of reasonably homogeneous tea bushes. Studies of the variation in chemical composition within the species are therefore best made on individual bushes, or on clones raised from cuttings of individual bushes. From the material available some twenty bushes and clones (referred to by their code numbers) have been chosen representing a wide range of botanical types.

Chromatograms have been run on reconstituted juices from these sources and typical results are given in Table 6. Each source has been examined several times throughout the season; the results quoted represent samples taken at about the same season in the year. 2/2/23, 14/4/23, 16/10/22 and 107/6 are bushes having much the same pattern of polyphenols as the average, although minor differences are apparent. 107/6 is unusual in containing trace amounts of both galloyl esters corresponding to spots 5 and 7.

Distinct abnormalities are shown by the other two sources. In 14/3/32 spot 9 (catechin) and spot 3 (DL-gallocatechin) are more abundant than spots 10 and 2 (the corresponding epimers), which is the reverse of what is normally found, while spot 5 (gallo-

Table 6. *Relative proportions of polyphenols in leaf from individual bushes as judged by chromatography*

(Sheets were sprayed with $FeCl_3$. Colour intensity is indicated by the same convention as in Table 1. Spot numbers as in Figs. 1 and 2.)

Bush no. ...	2/2/23	14/3/32	14/4/23	14/6/28	16/10/22	107/6
Date (1950)	29 May	29 May	6 June	31 May	29 May	30 June
Spot no.						
1	+ + +(+)	+ + + +	+ + +	+	+ + +(+)	+ + + +
2	+ +(+)	+(+)	+ + +	+ + +(+)	+ + +	+ +
3	(+)	+ +	+	+	+(+)	+
4	+	+(+)	(+)	+	+	(+)
5	(+)?	+ +	.	.	(+)?	(+)
6	+ + + +	+ + +(+)	+ + + +	+ + + +	+ + + +	+ + + +
7	.	.	.	.	.	(+)
8	+ +(+)	+ +	+ +	+ +	+(+)	+ +(+)
9	(+)	+(+)	+	+	+	(+)
10	+ +	+	+ +(+)	+ +	+(+)	+(+)

catechin-a gallate), which is normally absent or present in trace amounts only, is present in appreciable amounts. In 14/6/28 the *m*-digallic acid spot (1) is unusually weak. Apart from these two particular cases there are no major deviations from the normal distribution of polyphenols in the sources so far investigated.

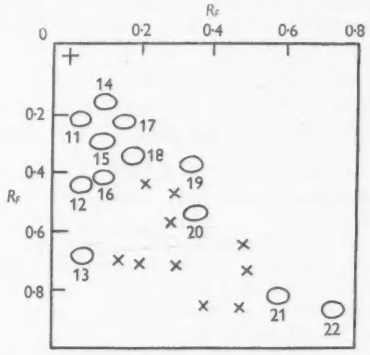

Fig. 3. Map showing location of unidentified polyphenols (11–22) occurring in tea-leaf juice. The crosses indicate positions occupied by identified polyphenols (cf. Figs. 1 and 2). Colour reagent $FeCl_3$.

Rather more variation appears amongst the minor, uncharacterized polyphenols. Fig. 3 shows the positions of twelve of these unidentified spots in relation to the identified catechins. Of these no. 20 gives a greenish-grey colour with ferric chloride, while all the remainder give dark blue-grey colours. None of these substances appears to be of general occurrence in tea as on many chromatograms they are entirely absent.

Considering spots 11–18 as a group, different sources of leaf differ markedly in the number and intensity of the spots on their chromatograms. The pattern for any given source is, on the whole, reproducible throughout the season. Bush 2/5/23 shows traces only of from two to four spots in this group, one of which is usually no. 14. Bushes 14/3/32 and 14/5/18 show as many as six spots, some of appreciable strength. The predominant spot in bushes 14/5/18, 16/10/22 and 107/6 is no. 14. In bush 124/30/33 spots 11 and 14 are of equal intensity, while in bush 14/3/32 spot 16 is usually rather stronger than spot 14. Bushes 2/2/23, 14/4/23 and 14/6/28 contain traces only of spots in this group. The overall intensity of these spots is increased in December, i.e. at the end of the growing season, when spot no. 14 in bush 14/3/32 attains medium strong intensity.

Spots 19 and 20 are of much more sporadic occurrence, and cannot be associated with any particular source.

Spots 21 and 22 are only very rarely observed. Both are present on several chromatograms of bush 14/4/23, and no. 21 alone sometimes occurs in bush 14/3/32.

Polyphenol distribution in the tea shoot

To determine the variation in polyphenol distribution from one part of the shoot to another, shoots of three leaves and a bud were divided into the following five portions: (1) bud plus first leaf, (2) second leaf, (3) third leaf, (4) upper stem, down to the point of attachment of the second leaf, (5) lower stem, below the second leaf. Chromatograms (Table 7) were compared of these five portions, and of hard, senile leaves gathered from below the plucking surface of the bush. Progressing from the youngest leaves to the more mature leaves, the two gallocatechins both increase in strength, whereas *m*-digallic acid, gallic acid, L-*epi*gallocatechin gallate and L-*epi*catechin gallate all decrease in strength. With respect to these six components the upper stem is intermediate between the third leaf and the senile leaf, while the lower stem is merely a weaker edition of the upper stem. Catechin and L-*epi*catechin appear to be most abundant in the second leaf and the upper stem, decreasing slightly in both younger and older leaves.

Table 7. *Comparison of chromatograms made on juice from different parts of the tea shoot*

(Chromatograms sprayed with $FeCl_3$ and colour intensities indicated as in Table 1. Spot numbers as in Figs. 1 and 2.)

Spot no.	Bud and 1st leaf	2nd leaf	3rd leaf	Upper stem	Lower stem	Senile leaf
1	+ + +	+ +(+)	+ +	+(+)	+	(+)
2	+ +	+ + +	+ +(+)	+ + +	+ +	+ + +(+)
3	+	+(+)	+(+)	+ +	+	+ +
4	+ +	+	+	(+)	(+)	(+)
5	.	.	.	.	.	.
6	+ + + +	+ + +(+)	+ + +	+ + +	+ +	+ +(+)
7	.	.	.	.	.	.
8	+ +	+ +	+(+)	+	.	+
9	(+)	+	(+)	+	(+)	(+)
10	+	+ +	+(+)	+ +	+	+(+)

Table 8. *Chromatograms of juice at successive stages of enzymic oxidation*

(Spot numbers as in Figs. 1 and 2. Colour reagent $FeCl_3$. Colour scale as in Table 1.)

Spot no.	Percentage oxidation				
	0	10	45	70	83
1	+ +	+ +	+ +	+ +	+(+)
2	+ +	+ +	.	.	.
3	(+)	(+)	(+)	.	.
4	(+)	+	+(+)	+ +	+
5	.	.	.	.	.
6	+ + +	+ + +	+	.	.
7	.	.	.	.	.
8	+ +	+ +	+ +	+(+)	+
9	+	+	+	(+)	(+)
10	+ +	+ +	+ +	+(+)	+

Table 9. *Chromatograms of teas fermented for different lengths of time*

(Colour reagent $FeCl_3$. Spot numbers as in Figs. 1 and 2 and colour scale as in Table 1.)

Spot no.	Withered leaf	Time fermented (hr.)			
		2	3	4	5
1	+ + + +	+ + + +	+ + + +	+ + +	+ +(+)
2	+ + +	.	.	.	.
3	+ +	(+)	.	.	.
4	+	+ +(+)	+ + +	+ + +	+ +
5	.	.	.	.	.
6	+ + + +	+ +	+	(+)	(+)
7	.	.	.	.	.
8	+(+)	+(+)	+	(+)	(+)
9	(+)	+	(+)	.	.
10	+(+)	+ +	+	(+)	(+)

Effect of fermentation

Fresh juice. Uncentrifuged juice (with the oxidase-containing chloroplasts still in suspension) is measured into a series of Warburg vessels and oxygen uptakes recorded in the usual way. At intervals one vessel is removed and the contents immersed in boiling water to stop fermentation. The last vessel is allowed to oxidize to completion, so that from the oxygen uptakes the percentage oxidation in each vessel can be calculated. Chromatograms are then run of the contents of selected vessels with the results shown in Table 8. L-*epi*Gallocatechin is the first catechin to become completely oxidized followed by DL-gallocatechin and L-*epi*gallocatechin gallate, while L-*epi*catechin gallate, L-*epi*catechin and catechin remain unaffected until more than half the total oxygen consumed in fermentation has been taken up. *m*-Digallic acid appears to be almost unaffected throughout fermentation while gallic acid shows a marked increase in the earlier stages of fermentation.

Fermenting tea leaf. Samples of fermented tea leaf were fired at varying time intervals from the start of rolling, and chromatograms run on 'reconstituted juices' from these samples. The effect of varying time of fermentation on the chromatogram is shown in Table 9. As with juice, the two gallocatechins and L-*epi*gallocatechin gallate are the first to be oxidized. *m*-Digallic acid is practically unaffected while gallic

acid increases considerably. After 4 hr. the chromatogram shows catechins in only trace amounts, but both gallic and *m*-digallic acids remain strong. Both acids decrease appreciably in strength if fermentation is further prolonged.

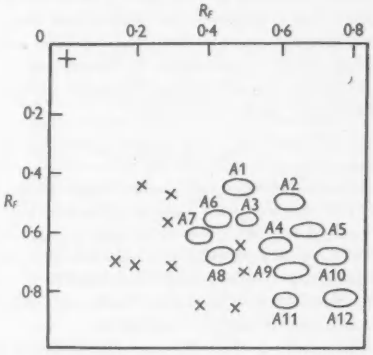

Fig. 4. Map showing location of the chief anthoxanthins found in tea-leaf juice. Crosses indicate positions of identified polyphenols (1–10). Colour reagent, NH_3 vapour.

Associated with a decrease in the intensities of the individual spots, there is a marked increase in the intensity, after spraying with ferric chloride, of a rather diffuse area at and near the origin. This diffuse area is probably to be identified with the various condensation products (cf. Harrison & Roberts, 1939) formed from the initial oxidation products of the catechins. In the earlier stages of fermentation three well defined spots corresponding with spots 11–13 of Fig. 3 are invariably encountered, whether or not they are present in the original green leaf. The significance of their apparent production during fermentation is not clear.

Chromatograms of fermented teas have never shown any detectable changes in the spots for anthoxanthins, suggesting that these substances undergo little or no chemical change during fermentation.

DISCUSSION

Our results indicate that partial epimerization of L-*epi*catechin to catechin takes place as a result of moderately long periods of boiling tea-leaf juice or infusion, as in the preparation of catechins from tea leaf. The presumption is that spots 2 and 3 on our chromatograms form a similar pair of epimers, and the gain in strength of spot 3 at the expense of spot 2, as a result of boiling, is due to an epimeric change. The conversion of one gallocatechin into another on boiling is strong supporting evidence for the view advanced by Bradfield & Bate-Smith (1950) that the L-gallocatechin and DL-gallocatechin isolated from tea by Bradfield *et al.* (1947) are in fact diastereoisomers.

L-*epi*Gallocatechin gallate and gallocatechin-a gallate form another pair of interconvertible substances, and, as already suggested by Bradfield & Bate-Smith (1950), the latter substance may possibly be the galloyl ester of gallocatechin and not *epi*gallocatechin. The R_F values of these two substances, however, are almost identical in *n*-butanol-acetic acid, but are appreciably different in phenol. As the epimeric pairs so far considered have almost identical R_F values in phenol but appreciably different values in *n*-butanol-acetic acid, there is some uncertainty in characterizing the two gallocatechin gallates as simple epimers. The retention of the name gallocatechin-a gallate therefore serves to indicate that the evidence is not yet sufficiently precise to conclude that this substance differs from L-*epi*gallocatechin gallate only in the configuration of the substituted hydroxyl group in the 3-position.

The formation of the catechin corresponding to spot 7 from L-*epi*catechin gallate on boiling, and the relative positions of spots 7 and 8 on the chromatogram, suggest that this catechin bears the same relation to L-*epi*catechin gallate that gallocatechin-a gallate does to L-*epi*gallocatechin gallate. We propose therefore to refer to this substance as catechin-a gallate. The existence of this substance does not appear to have been established before.

It follows from the above results that the extract used by Bradfield (1946) in his approximate analysis of the catechins of Ceylon green tea does not give a true picture of the catechins and other polyphenols in the green leaf. The method of extraction leads to a considerable modification of the relative proportions of the epimers of the simple catechins, and the transformation products of galloyl esters, represented on the chromatograms by spots 5 and 7, are normally artifacts, as these substances are only rarely detectable in fresh tea-leaf juice. Further, an important polyphenol, possibly identical with *m*-digallic acid, is not appreciably extracted from the infusion by ethyl acetate, so that its occurrence in tea was not recognized.

We may conclude that the so-called tea tannin is a mixture of eight catechins, traces of gallic acid and appreciable amounts of what is possibly *m*-digallic acid, together with a few other polyphenols normally occurring in trace amounts only. There is no evidence for the presence of simple polymers of catechins, as suggested by Harrison & Roberts (1939), so that this early suggestion is now withdrawn. This conclusion has an important consequence when considering analytical methods for the determination of tannin in tea. Not all of the substances concerned are precipitated by gelatin, even in the presence of acid and salt, so that the estimation of tea tannin by Löwenthal's method, as

described by the Association of Official Agricultural Chemists (1945), and by Barua & Roberts (1940), is fundamentally wrong. We have been unable to find any non-phenolic component of the tea leaf oxidizable by potassium permanganate under the conditions of the Löwenthal titration so that the non-tan titre may probably be taken as a measure of the polyphenols not precipitated by gelatin. There is apparently, therefore, no point in carrying out the gelatin precipitation, and the total potassium permanganate titre is considered a reasonably good estimate of total polyphenols (including catechins) in unoxidized tea leaf. Procedures involving oxidation by alkaline iodine and alkaline potassium permanganate, also described by Barua & Roberts (1940), cannot give precise values for total polyphenols, whether the non-tan separation is carried out or not, as under alkaline conditions, substances other than polyphenols (glucose, etc.) are oxidized by iodine and potassium permanganate. On account of the complications introduced by condensation during fermentation, the alkaline potassium permanganate method of Barua & Roberts (1940) still gives the best estimate of total polyphenols in fermented tea, but it is now emphasized that the titre is only strictly proportional to these polyphenols so long as a constant proportion of the polyphenols is precipitated on treatment with gelatin. For most teas this appears to be approximately the case (unpublished observations), but it is emphasized that all oxidimetric methods of titration for polyphenols in tea give results which need to be interpreted with considerable care.

The successive oxidation of substrates during fermentation, as indicated by chromatography, is a useful confirmation of earlier manometric work (Roberts & Wood, 1950). The appearance of gallic acid indicates that the galloyl esters are hydrolysed as well as oxidized during fermentation. Neither gallic acid nor *m*-digallic acid are oxidized rapidly by the tea-oxidase, hence their persistence in fermented tea.

The establishment of the presence of these two acids in fermented tea shows that complete oxidation of polyphenols cannot normally be realized in fermentation. After catechins have been completely oxidized a slow uptake of oxygen will persist for many hours owing to the slow oxidation of these polyphenolic acids, and it will be difficult to state just when oxidation is complete. The estimation of the percentage oxidation of substrates in partly fermented tea by the residual uptake method (Roberts, 1941) is therefore a rather less precise method than was originally thought, although it still remains a useful technique.

SUMMARY

1. Chromatograms of tea-leaf juice show both epimers of catechin and gallocatechin together with their galloyl esters. Gallic acid and a polyphenolic acid believed to be *m*-digallic acid are also present.

2. The same chromatogram sometimes reveals traces of other polyphenols, while anthoxanthins and organic acids are also detected.

3. The boiling of a tea-leaf infusion results in epimerization of the simple catechins and a transformation, which may be epimerization, of the galloyl esters. Estimations of the relative abundance of catechins in tea must therefore be carried out on freshly expressed juice.

4. The variations in the relative proportions of the catechins and polyphenolic acids in different individual bushes and in different parts of the shoot are studied.

5. During fermentation the various catechins are oxidized successively rather than simultaneously. Gallic acid is liberated during fermentation. The chromatogram of fully fermented tea shows no catechins, but considerable amounts of gallic and *m*-digallic acids.

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The Direct Colorimetric Estimation of Reducing Sugars and other Reducing Substances with Tetrazolium Salts

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Since Kuhn & Jerchel (1941) showed that tetrazolium salts were reduced by bacteria, yeasts and germinating seeds, 2:3:5-triphenyltetrazolium chloride or bromide has come into prominence as a viability testing reagent (Cottrell, 1947) and for vital staining (Mattson, Jensen & Dutcher, 1947). Its specific advantages for this purpose and for the purposes which follow lie in its E_0 value of $-0{\cdot}08$ V. (Jerchel & Mohle, 1944) and, more especially, in the fact that it is colourless in the oxidized form and an intense cherry red colour in the reduced form. This latter peculiarity gives it a measure of uniqueness among organic compounds.

In oxidized form the compound is soluble in water; after reduction, which with sugars only takes place in alkaline solution, it becomes insoluble in water but soluble in many organic solvents. Recently Mattson & Jensen (1950) have described its use in a method for the estimation of reducing sugars. The advantage of such a method is obvious as the substitution of a direct colorimetric method for titration or for an indirect colorimetric or gravimetric method can lead to improved accuracies and a saving of time.

Apart from a general investigation of the properties and behaviour of the reagent, four possible specific applications were chosen—estimation of reducing sugars, of blood sugar, of cysteine and of ascorbic acid. Before any specific methods could be elaborated for the colorimetric measurement of reducing substances it was necessary to establish the general characteristics and stoicheiometrical relations of the reagent in both oxidized and reduced forms.

EXPERIMENTAL

Stability

2:3:5-Triphenyltetrazolium bromide (tetrazolium) in aqueous solution must be kept in the dark (an amber bottle is quite satisfactory), and it has been found advisable to make up solutions weekly. Tetrazolium is reduced to triphenyl formazan (formazan) which, in aqueous *iso*propanol, is unstable in alkaline solution, but becomes stable after neutralization. In neutral solution no appreciable fading of colour could be measured in a photoelectric colorimeter after standing for an hour in ordinary room lighting. Undissolved triphenyl formazan is decomposed rapidly at 100° and more slowly at room temperature when in contact with aqueous alkali.

Solubility

Formazan was found to be soluble in light petroleum, benzene, toluene, xylene, methanol, ethanol, *n*-propanol, *iso*propanol, *n*-butanol, *iso*butanol, amyl alcohol, glacial acetic acid, acetone, ethyl ether, chloroform, carbon tetrachloride and pyridine.

Optimum conditions of reaction

In vivo, reduction of tetrazolium may take place at a pH of about 5·7 (e.g. germinating cereal grains). *In vitro*, Kun & Abood (1949) found reduction to take place in the presence of succinic dehydrogenase at pH 7·4, but state that in the absence of succinate, tissue homogenates do not reduce tetrazolium except under strongly alkaline conditions. It was found that maximum *in vitro* reduction of tetrazolium by glucose, lactose or cysteine was only obtained in strongly alkaline solutions and at 100°. Ascorbic acid under the same conditions caused rapid *in vitro* reduction at room temperature. In view of the instability of formazan in alkali, the minimum strength of alkali which would give sufficiently rapid colour development was chosen. Because simultaneous production and destruction of formazan take place, it was necessary rigidly to control time, temperature and pH to ensure stopping the reaction when maximum formazan production had taken place. Moreover, the time taken to achieve optimal production of formazan varies for any given reducing agent according to its concentration, but it was found possible to choose a suitable range of concentrations such that the time factor was constant for maximum formazan production. In Fig. 1 are curves relating amount of formazan produced to reduction time, the variable being glucose concentration. Similar curves were prepared for the

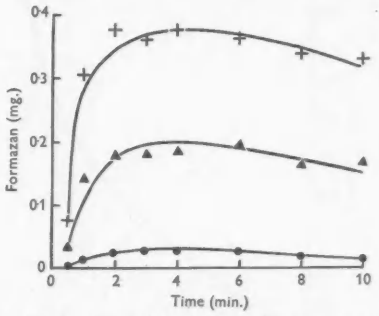

Fig. 1. Effect of reaction time on formazan produced. Glucose: ●—●, 0·2 mg.; ▲—▲, 1 mg.; +—+, 2 mg.

other reducing substances mentioned to establish optimum conditions for the reaction.

The amount of formazan produced in any specific reduction by sugar or other reducing agents bears no relationship to the quantity which would be expected from stoicheiometric considerations as alkaline destruction occurs concurrently with formation.

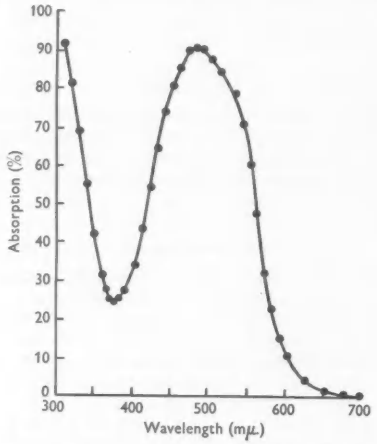

Fig. 2. Absorption curve of triphenyl formazan in *iso*propanol solution.

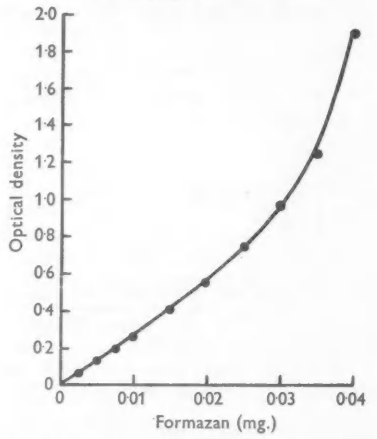

Fig. 3. Relation between absorption and concentration of formazan in *iso*propanol—Corning filter M515 in 5 mm. cell.

A sample of pure triphenyl formazan was prepared by the reduction of 1 g. of tetrazolium bromide by 1 g. of ascorbic acid dissolved in 25 ml. of distilled water + 5 ml. of 10% (w/v) NaOH at room temperature. After the reaction had proceeded for 20 min. the solution was neutralized, filtered and the triphenyl formazan washed on the filter paper and dried at 37°. It was used to determine the extinction coefficient and spectral absorption of formazan, using the Beckman spectrophotometer. Maximum absorption of triphenyl formazan in *iso*propanolic solution was found to be at 485 mμ.

The $E^{1\,cm.}_{0{\cdot}01\,\%}$ 485 mμ. in *iso*propanol was found to be 5. The absorption curve is given in Fig. 2.

Formazan in solution obeys Beer's Law. Fig. 3 is a graph relating concentration to optical density using Corning filter M515 in the Lumetron colorimeter (5 mm. cell). Using Ilford filter 602 in the Spekker absorptiometer (1 cm. cell) the relationship between concentration and drum reading was found to be perfectly linear.

Method for glucose and lactose

Standard curve. Into clean dry 6 × 0·75 in. test tubes graduated at 20 ml. are pipetted dilutions of glucose or lactose to give a range of 0·2–2 mg. and the volume adjusted to 3 ml. with distilled water. In each series a blank is included containing 3 ml. distilled water. To each tube is added 1 ml. of 1% (w/v) solution of triphenyltetrazolium bromide ('Grodex', May and Baker) and 2 ml. N-NaOH. The tubes are heated in a boiling-water bath for exactly 3 min., and on removing from the bath, 2 ml. of approximately 1·1 N-acetic acid are added and the tubes immediately cooled in a cold-water bath. The volume is adjusted to 20 ml. with *iso*propanol and the mixture shaken to dissolve the formazan. Into another graduated test tube 2 ml. is pipetted, and the volume adjusted to 20 ml. with *iso*propanol. Colour measurement is made with a suitable colorimeter.

Standard concentration/extinction curves for glucose and lactose are similar to that shown for ascorbic acid in Fig. 5, and may be used for the determination of unknown concentrations of these sugars.

Method for determination of blood sugar

The method is suitable for the determination of glucose in blood, but because of the small quantities to be determined some modifications are required. Using the Lumetron colorimeter, tubes graduated at 20 ml. are required, whilst for the Spekker absorptiometer tubes graduated at 25 ml. are necessary. A suitable range of dilutions of standard is from 0·02 to 0·2 mg. glucose per 2 ml. The blank tube contains 2 ml. of distilled water.

To each tube add 1 ml. of 0·3% (w/v) triphenyltetrazolium bromide and 1 ml. 2 N-NaOH. Heat in boiling-water bath for exactly 3 min. On removal acidify with 1 ml. of 2·1 N-acetic acid. Cool in cold-water bath and adjust volume with *iso*propanol. A typical standard curve is shown in Fig. 4, together with a curve for lactose estimated under the same conditions.

Benzoic acid used to preserve standard solutions of glucose has no appreciable effect on the production of formazan. For the determination of blood glucose, a 0·2 ml. sample of blood is taken and the protein removed by the method of Somogyi (1945) as follows. The sample of blood is laked in 3 ml. distilled water and 0·4 ml. 0·3 N-$Ba(OH)_2$ added followed by 0·4 ml. 5% (w/v) $ZnSO_4.7H_2O$. The mixture is shaken, warmed and filtered. Accuracy of the concentration of the reagents is less important than the requirement that the

Table 1. *Blood sugar determinations by tetrazolium method and Hagedorn & Jensen method*

	Tetrazolium method		Method of Hagedorn & Jensen	
	Glucose (mg./100 ml.)	Glucose recovered (mg./100 ml.)	Glucose (mg./100 ml.)	Glucose recovered (mg./100 ml.)
Initial blood sugar	79		78	
Glucose added (mg./100 ml.):				
20	97	18	96	18
40	117	38	117	39
60	139	60	133	55
80	158	79	155	77
100	177	98	174	96

alkali must neutralize the $ZnSO_4$ precisely, volume for volume, using phenolphthalein as indicator. Filtrate (2 ml.) equivalent to 0·1 ml. of blood is pipetted into a graduated test tube, and 1 ml. of the tetrazolium solution and 1 ml. of 2 N-NaOH added. The tube is then treated in the same way as the standard tubes. The intensity of colour is measured as for glucose.

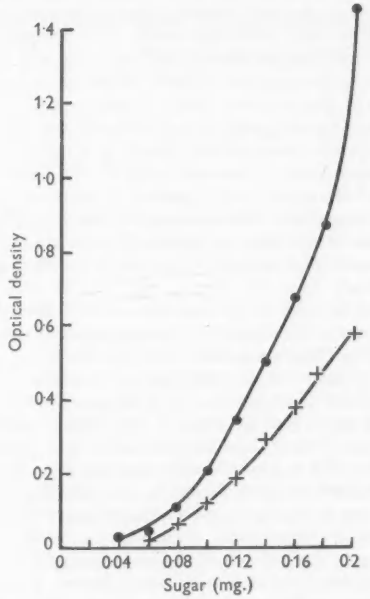

Fig. 4. Standard curves by blood-sugar method. ●—●, glucose; +—+, lactose.

Table 1, showing a comparison between the tetrazolium method and that of Hagedorn & Jensen in determining glucose added to whole blood, indicates that good agreement is obtained between the two methods. The results are the mean of duplicate determinations. In addition, the tetrazolium method proved much simpler and quicker in operation.

Method for ascorbic acid

Since the reaction between ascorbic acid and tetrazolium is extremely rapid at 100°, and difficulties due to the different rates of production of formazan at varying concentrations of ascorbic acid, and the destruction of formazan by alkali, arise, this reaction is best carried out close to room temperature and, to prevent reduction by light, in the dark.

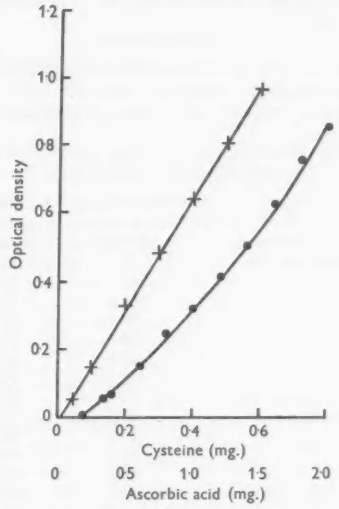

Fig. 5. Standard curves for ascorbic acid, ●—●, and cysteine, +—+.

Standard solutions of ascorbic acid are prepared in 3% (w/v) metaphosphoric acid and are used immediately after preparation. The method follows that of glucose with the difference that the time of reaction is 20 min. at room temperature, or preferably at 37° (incubator) and in the dark. To neutralize the HPO_3, 2 ml. of 2 N-NaOH are used instead of N-NaOH. As dilution with *iso*propanol causes precipitation of phosphates, it is necessary to add 5–6 ml. distilled water before adjusting the volume with *iso*propanol. Alternatively, the formazan may be extracted from the

reaction tubes with an immiscible solvent, the volume being adjusted in the usual way.

Fig. 5 relates a range of 0·2–2 mg. of ascorbic acid to optical densities. An attempt was made to determine the ascorbic acid content of fruit juices by this method, but owing to the presence of other strongly reducing substances no comparison with results determined by the 2:6-dichlorophenolindophenol method could be obtained.

Method for cysteine

Cysteine reacts with tetrazolium much more slowly than glucose or ascorbic acid and the optimum time of reaction under similar conditions is 20 min. at 100°. Moreover, prolonged heating of the formazan produces conglomerates which are difficult to dissolve. It was found advisable to use a more dilute solution of tetrazolium (0·2 %, w/v) which, with amounts of cysteine ranging from 0·05 to 0·6 mg., has an optimum reaction time of 25 min. The method is otherwise similar to that for glucose. After acidification the volume is adjusted to 20 ml. with *iso*propanol. No further dilution is necessary. A typical curve is shown in Fig. 5.

Analytical results

Results obtained by the various modifications of the basic method which have been described above are shown in Table 2. Each result is the mean of three

Table 2. *Results of colorimetric estimations using triphenyltetrazolium bromide*

Substance	Concentration (mg./100 ml.) Found	Actual
D-Glucose	31·9	31·8
	41·7	39·4
	52·5	51·6
	60·4	59·7
Lactose	26·3	27·0
	40·1	40·6
	50·4	50·6
	59·4	60·9
Ascorbic acid	28·2	29·9
	40·2	41·0
	49·2	49·6
	59·9	60·4
Cysteine HCl	10·0	9·4
	19·5	20·0
	33·9	32·9
	46·8	48·1

single determinations made at widely spaced points on the 'standard' curves. Greater accuracy than is indicated here may be obtained, if necessary, by estimations carried out in duplicate or triplicate.

DISCUSSION

Tetrazolium is a new analytical reagent, which possesses several unique properties. In the first place it allows the replacement of volumetric methods by colorimetry, so replacing a subjective (titration) by an objective method (electrometric colorimetry). Secondly, by eliminating one stage from customary indirect methods it provides less latitude for error and gives increased speed. Thirdly, it could prove useful in reactions where it is necessary to extract the formazan from the reaction mixture with an immiscible solvent (chloroform, benzene) which would not extract any interfering water-soluble pigments which might be present.

The reagent lends itself to micro and ultra-micro estimations as the colour developed is very intense relatively to the material being estimated. If the alkaline destruction of the formazan could be prevented or reduced by the introduction of a stabilizer, e.g. cysteine, then this advantage could be even greater, but so far efforts in this direction have failed.

Though the method is quite empirical it is not different in this respect from many others which are in daily use. Whilst strict adherence to the standard conditions chosen is essential the conditions may be varied to suit particular cases. For example, the range of concentrations of reducing substances could be increased if suitable dilutions of the formazan solutions were made. However, it cannot be increased indefinitely under one set of standard conditions, as for each range of concentrations there is an optimum time of reaction, and if this time is exceeded the amount of formazan decomposed by the alkali increases. This is shown in the blood glucose curves where the conditions chosen are not the optimum for the whole range of concentrations necessary.

The method of Mattson & Jensen (1950) differs very markedly from the present method in several respects. The temperature used is $25 \pm 0{\cdot}1°$ which only permits the development of a small fraction of the colour which can be obtained, and hence lessens sensitivity. Furthermore, in this region, the temperature is very critical, and slight deviations can readily lead to errors of the order of 10 %. No such critical behaviour is found at 100° and the time of reaction is much shorter. Acetic acid is used to neutralize the sodium hydroxide as the buffering action of the acetate retards decomposition of the formazan if excess acid is added. Moreover, sodium acetate is sufficiently soluble in the miscible solvents to prevent precipitation, whereas, if sulphuric or hydrochloric acids are used, the sulphate or chloride formed tend to precipitate, making filtration necessary.

Acetone, methanol, ethanol or *iso*propanol may be used as solvents. *iso*Propanol was chosen because of its relatively low volatility and general convenience in use. It possesses several obvious advantages over pyridine, which was used by Mattson & Jensen (1950).

SUMMARY

1. A study has been made of the reaction between triphenyltetrazolium bromide and various reducing agents. Conditions for optimal production of formazan by this reaction were established.

2. Triphenylformazan has been prepared and its absorption and solubility characteristics determined.

3. Direct colorimetric methods for the estimation of blood sugar, glucose, lactose, cysteine and ascorbic acid based on the use of tetrazolium are described.

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The Nature and Mechanism of Action of the Enzymes Responsible for Tissue Respiration during Aseptic Autolysis

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It is known that tissues, isolated from organisms and maintained under aseptic conditions, undergo processes of autolysis, characterized by progressive hydrolysis of proteins brought about by the tissue cathepsins. The mechanism of catheptic action has been studied by several workers, and it has been shown that the activity of the cathepsins is stimulated by acids, thiols and cyanide. Less attention has been devoted to the action of other enzymes during autolysis. Edlbacher & Koller (1934) showed that necrotic tissue from Jensen's sarcoma is rich in arginase, and Bayerle (1936) extended the observation to necrotic non-cancerous tissues. Michelazzi (1947) studied the behaviour of the oxidative metabolism of tissues in conditions of aseptic autolysis, and obtained evidence that homogenates of guinea pig liver, kidney and brain can consume small amounts of oxygen even many days after the start of aseptic autolysis in the organs. Since boiling for 20 min. abolished the respiratory activity, Michelazzi concluded that this must be due to the persistence of certain oxidizing enzymes. He observed nevertheless that fumarate, lactate, aspartate, DL-alanine, phenylalanine and ethanol are not attacked by autolysing tissues and that cytochrome oxidase is completely inactive. The present work is also concerned with oxidative metabolism of the tissues in conditions of aseptic autolysis.

EXPERIMENTAL

Conditions of autolysis. Liver and kidney of guinea pigs, mice and rats were used. The organs were removed under sterile conditions from the bled animals and were placed in sterile vessels containing 30 ml. 0·067 M-acetate buffer (pH 4·5), which was then covered to a depth of 1 cm. with toluene. The vessels were closed with paraffined glass stoppers and were placed in a thermostat at 37·5° for periods of from 20 to 50 days. At the desired times the vessels were opened, the toluene removed, and the organs washed with 0·067 M-phosphate buffer (pH 7·4) blotted with filter paper, and weighed.

Homogenates were made by prolonged grinding in a mortar, the amounts of tissue used in each experiment being such that 0·5 g. of liver or 0·25 g. of kidney was contained in 3 ml. of the suspension. Controls for sterility were made by inoculation into broth, any contaminated specimens being discarded.

Measurement of respiration. Residual respiration was estimated by the Warburg technique over a period of 60 min. at 38° in 0·067 M-phosphate buffer (pH 7·4).

Succinoxidase activity was determined by the method of Schneider & Potter (1943), and lactate dehydrogenase by the method of Green & Brosteaux (1936): α-glycerophosphate dehydrogenase was studied by measuring the extra O_2 uptake in the presence of 0·2 ml. 0·2 M-sodium α-glycerophosphate (Hofmann-LaRoche). D- and L-Amino-acid oxidase activities were determined by the procedure of Rodney & Garner (1938), 0·2 ml. of DL- and L(+)-alanine (0·2 M) being used as substrates.

Monoamine oxidase activity was followed by measuring the extra O_2 uptake in the presence of 0·2 ml. of 0·25 M-tyramine hydrochloride or of 0·25 M-adrenaline hydrochloride (both from Hofmann-LaRoche), 0·1 ml. of 0·2 M-KCN being added to each flask; control flasks to allow for the autoxidation of tyramine and adrenaline were also included.

Inhibitors. NaCN and NaF, each in a final concentration of 0·05 M. Urea and hydrazine hydrate in final concentrations of 3 and 0·01 M, respectively.

sec.-Octyl alcohol, 0·05 ml./Warburg flask.

Preparation of monoamine oxidase. The preparation, obtained from calf liver by the method of Kohn (1937), gave a $Q_{O_2} = 12·6$ μl./mg. dry wt./hr. with tyramine as substrate. Storage at 0° for 7 days caused no significant loss of activity. The specificity of the purified enzyme was tested against the following substrates, none of which was attacked: DL-alanine, L(+)-alanine, sodium succinate, sodium lactate, sodium fumarate, sodium α-glycerophosphate, histamine hydrochloride.

Detection of amines. Amines were detected in the tissue autolysates by measuring the extra O_2 consumption induced by incubating samples of the homogenates with the purified calf-liver monoamine oxidase preparation.

Ammonia production was measured by the procedure used by Thompson & Tickner (1949).

Hydrogen peroxide production during the oxidation of tyramine was demonstrated (1) by observing the secondary oxidation of ethanol to acetaldehyde (Keilin & Hartree, 1936; Kohn, 1937), 1 mg. of ethanol being placed in the side arms of the Warburg flasks, and tipped into the main compartment 30 min. after the first reading; (2) by the oxidation of haemoglobin to methaemoglobin (Bernheim & Michel, 1937) by the following technique: at the end of the measurement of O_2 uptake in the presence of tyramine, the contents of the Warburg flasks were filtered; to the filtrate was added 0·5 ml. of a very dilute haemoglobin solution (obtained by lysis of guinea pig erythrocytes), and the mixture was allowed to stand for 2–4 hr.; in a second flask 0·5 ml. of the haemoglobin solution was added to 3 ml. 0·067 M-phosphate buffer (pH 7·4) as a control. The production of a brown colour in the first flask was accepted as proof of the presence of traces of H_2O_2.

Aldehyde production by the autolysing tissues was detected by adding to the homogenates a purified xanthine oxidase preparation, previously placed in the side arm of the Warburg flasks, and by measuring for 60 min. the extra O_2 uptake. These experiments were carried out without cyanide, which strongly inhibits xanthine oxidase. The xanthine oxidase was prepared from cream from unpasteurized milk by the method described by Ball (1939). Its activity was tested by studying the oxidation of acetaldehyde at 38° and at pH 7·5.

RESULTS

Residual respiration of autolysing tissues

The results of Michelazzi (1947), showing that homogenates of autolysing liver and kidney take up small quantities of O_2 even many days after the beginning of autolysis, have been confirmed (see Table 1).

In Table 1, since the observed O_2 uptakes were small in relation to the amount of tissue employed, they are expressed as μl. O_2/hr. rather than as Q_{O_2} values.

Table 1. *Residual oxygen uptake of liver and kidney homogenates during aseptic autolysis*

(Values are expressed as μl. O_2/hr. Gaseous environment, air. Temp., 38°.)

No. of exp.	Animal	Time of autolysis (days)	O_2 consumed in 1 hr. (μl.)
		Liver	
1	Guinea pig	15	30·7
1	Guinea pig	17	30·6
2	Guinea pig	18	27·6
1	Guinea pig	20	27·5
3	Guinea pig	23	34·4
1	Guinea pig	25	20·4
2	Guinea pig	30	0·8
1	Guinea pig	40	0
1	Guinea pig	50	0
2	Rat	15	32·3
2	Rat	20	21·8
2	Rat	22	28·6
4	Rat	25	25·6
2	Rat	30	11·0
1	Rat	40	0
2	Mouse	20	12·4
2	Mouse	25	10·6
		Kidney	
1	Guinea pig	17	20·5
3	Guinea pig	20	20·0
1	Guinea pig	25	4·2
1	Guinea pig	30	4·1
1	Guinea pig	40	0
1	Rat	15	30·5
2	Rat	20	21·0
4	Rat	25	11·5
1	Rat	26	10·3
2	Rat	30	2·7
1	Rat	50	0

Residual O_2 uptake is always small, being maximal in the first days of autolysis and then progressively decreasing. A small quantity of O_2 is consumed even after 20–30 days' autolysis; in course of time the residual respiration is completely suppressed. Residual O_2 uptake is generally higher in the liver than in the kidney; no large differences exist between guinea pig, rat and mouse in the values for liver, although those obtained with rat kidney are generally lower than those with guinea pig kidney.

Marked quantitative differences were observed among the various experiments as had also been noticed in Michelazzi's experiments.

The residual respiration of guinea pig liver was abolished completely by boiling for 2 min., and was markedly decreased by heating at 60° for 10 min.

Influence of pH *on residual respiration*

Measurements of residual respiration were carried out at pH 6·8, 7·0, 7·4, 7·6 and 7·8. It was found that the optimum pH for residual O_2 uptake of these homogenates of autolysing tissue is 7·4.

Table 2. *Influence of oxygen tension on residual respiration of autolysing tissue homogenates*

Animal	Organ	Time of autolysis (days)	O_2 consumed (μl.) In air	O_2 consumed (μl.) In 100% O_2	Increase (%)
Guinea pig	Liver	20	57·4	69·2	20·5
Guinea pig	Liver	21	25·0	34·1	36·2
Guinea pig	Liver	22	16·0	26·5	54·9
Guinea pig	Liver	22	15·5	21·8	39·8
Rat	Liver	22	7·6	14·3	88·3
Rat	Kidney	22	5·3	8·6	63·5

Table 3. *Influence of some inhibitors on residual respiration of autolysing tissue homogenates*

Animal	Organ	O_2 consumed (μl./hr.)	With KCN: O_2 consumed (μl./hr.)	With KCN: Inhibition (%)	With NaF: O_2 consumed (μl./hr.)	With NaF: Inhibition (%)	With *sec.*-octyl alcohol: O_2 consumed (μl./hr.)	With *sec.*-octyl alcohol: Inhibition (%)
Guinea pig	Liver	27·5	25·7	6·29	26·4	3·89	0	100
Guinea pig	Liver	29·0	26·3	9·24	29·1	0	3·5	87·96
Guinea pig	Kidney	16·4	15·6	5·05	15·9	3·16	1·1	87·51
Rat	Liver	21·8	21·1	3·48	21·6	1·00	2·3	86·84
Rat	Kidney	20·5	20·0	2·48	19·3	6·09	3·6	82·46

Influence of oxygen tension on residual respiration

The O_2 uptakes of the homogenates in air and in 100% O_2 were compared. The results are summarized in Table 2.

From Table 2 it can be seen that pure O_2 increases the rate of residual respiration of autolysing tissue homogenates. The actual increase is variable, ranging from 36 to 88%.

Action of inhibitors on residual respiration

The results obtained with certain enzyme inhibitors are shown in Table 3. It was found that cyanide and fluoride exert only a very weak effect on the residual O_2 uptake, which is, however, strongly inhibited by *sec.*-octyl alcohol. The weak inhibitory action of cyanide confirms the complete ineffectiveness of cytochrome oxidase in the autolysing tissues (Michelazzi, 1947) and excludes the possibility of residual O_2 uptake being due to the activity of oxidases acting through the cytochrome-cytochrome oxidase system.

Oxidative activity of autolytic tissues upon certain substrates

The autolysing tissues were tested for content of various enzymes; succinoxidase (acting through the cytochrome-cytochrome oxidase systems), lactic dehydrogenase, α-glycerophosphate dehydrogenase and D- and L-amino-acid oxidases. None of these was detected.

Since none of these enzymes maintains its activity in the autolytic tissues, it seemed likely that the enzyme responsible for the residual O_2 uptake in autolysing tissues belongs to the group of oxidases which can directly transfer hydrogen to atmospheric O_2.

Among this group is monoamine oxidase discovered by Hare (1928) and fully described by Pugh & Quastel (1937), Kohn (1937), and Blaschko, Richter & Schlossmann (1937). Monoamine oxidase is very abundant in guinea pig liver, brain and kidney and in rat liver and brain, while it is very scarce in the rat kidney (Pugh & Quastel, 1937). It was also recently demonstrated in human placenta by Thompson & Tickner (1949). No inhibition of its activity is caused by cyanide or fluoride, although it is strongly inhibited by octyl alcohol and by 3M-urea; 0·01M-hydrazine hydrate also inhibits this enzyme.

Inhibitory action of 3M-urea and of 0·01M-hydrazine hydrate on residual oxygen uptake by the tissues during aseptic autolysis

The effect of 3M-urea and of 0·01M-hydrazine hydrate (accurately neutralized) on residual respiration of the autolysing tissue homogenates was next tested. The results, which are shown in Table 4, indicate that both those compounds in these concentrations are strongly inhibitory.

Increase of oxygen uptake on addition of tyramine and adrenaline to autolysing tissue homogenates

From the preceding experiments it appeared probable that residual O_2 uptake of the autolysing tissues was due to the activity of monoamine oxidase. Experiments were therefore carried out to study the effect of addition of tyramine and adrenaline on the residual O_2 uptake of the autolysing tissues.

The results which were obtained are presented in Table 5. Addition of tyramine strongly increases the O_2 uptake of the homogenates. Adrenaline also produces an increase, but much lower than that obtained with tyramine. This fact is in agreement with

Table 4. *Action of* 3 M-*urea and of* 0·01 M-*hydrazine hydrate on the residual oxygen uptake of autolysing tissue homogenates in the guinea pig*

(Values expressed as μl. O_2/hr.)

Organ	Time of autolysis (days)	O_2 consumed in 1 hr. (μl.)	O_2 consumed in 1 hr. in the presence of inhibitor (μl.)	Inhibition (%)
		Urea (3 M)		
Liver	20	27·5	14·1	48·7
Liver	22	32·0	11·5	64·15
Kidney	20	22·4	9·0	58·8
Liver	20	21·8	8·2	62·2
Kidney	20	10·5	2·2	78·7
		Hydrazine hydrate (0·01 M)		
Liver	23	14·9	2·0	86·8
Kidney	23	9·8	4·2	56·8
Liver	20	24·4	11·2	53·8
Kidney	20	16·0	9·2	42·6

Table 5. *Oxidation of tyramine and adrenaline* (0·25 M) *by homogenates of autolysing liver and kidney*

Animal	Time of autolysis (days)	O_2 consumed in 1 hr. (μl.)	With tyramine: O_2 consumed in 1 hr. (μl.)	With tyramine: Increase	With adrenaline: O_2 consumed in 1 hr. (μl.)	With adrenaline: Increase
			Liver			
Guinea pig	5	80·6	208·2	+128·53	100·4	+19·75
	18	22·4	110·0	+ 87·63	38·9	+16·57
	18	12·8	83·2	+ 70·34	19·4	+ 6·59
	25	17·5	75·4	+ 57·86	25·3	+ 7·81
	20	25·0	42·6	+ 17·54	—	—
	21	40·1	79·2	+ 39·03	47·1	+ 6·98
Rat	15	38·8	117·9	+ 79·07	—	—
	25	37·6	52·7	+ 15·15	—	—
	25	10·6	64·9	+ 54·34	14·2	+ 3·63
	10	62·7	67·8	+ 5·12	68·2	+ 5·44
	12	42·4	—	—	51·2	+ 8·77
	15	52·0	55·6	+ 3·65	—	—
Mouse	20	12·4	25·7	+ 13·36	—	—
			Kidney			
Guinea pig	10	10·5	59·8	+ 49·32	42·6	+32·11
	20	21·5	38·9	+ 17·35	—	—
	25	20·7	34·7	+ 14·06	25·3	+ 4·66
	20	17·4	40·3	+ 22·91	24·1	+ 6·70
Rat	35	7·2	8·4	+ 1·18	—	—
	25	7·1	16·6	+ 9·44	9·4	+ 2·34
	25	16·5	18·6	+ 2·12	—	—
	22	3·0	12·3	+ 9·28	7·4	+ 4·38
	20	20·1	28·7	+ 8·63	22·4	+ 2·26

the findings of Pugh & Quastel (1937) and of Kohn (1937), primary amines being oxidized more readily than the secondary amines.

Increase of O_2 uptake on addition of tyramine was higher in guinea pig than in rat tissues; the values observed in the rat kidney were, in general, very low. These facts also are in agreement with the claims of Pugh & Quastel (1937), of Kohn (1937) and Schales (1947), oxidation of amines taking place very slightly in rat kidney.

The influence of O_2 tension on the oxidation of tyramine by autolysing tissue homogenates was also investigated.

The results, which are summarized in Table 6, show that 100% O_2 markedly increases the rates of oxidation of tyramine by autolysing tissue homogenates; this fact agrees with the statements of Kohn and of Thompson & Tickner (1949), who observed that monoamine oxidase activity is a function of O_2 tension.

Table 6. *Influence of oxygen tension on the oxidation of tyramine by homogenates of autolysing liver and kidney*

			Air			100% O_2		
				With tyramine			With tyramine	
Animal	Organ	Time of autolysis (days)	O_2 consumed in 1 hr. (μl.)	O_2 consumed in 1 hr. (μl.)	Increase	O_2 consumed in 1 hr. (μl.)	O_2 consumed in 1 hr. (μl.)	Increase
Guinea pig	Liver	20	27·4	39·2	+11·77	43·7	64·7	+21·00
Guinea pig	Liver	25	25·0	42·6	+17·54	34·1	55·5	+21·38
Guinea pig	Liver	22	16·0	27·7	+11·70	26·5	48·4	+21·93
Guinea pig	Kidney	25	5·5	25·6	+20·05	21·8	52·6	+30·86
Rat	Liver	20	7·6	60·1	+52·49	14·3	93·3	+78·93
Rat	Kidney	20	5·3	18·0	+12·78	8·6	33·4	+24·83

Table 7. *Increase by addition of purified monoamine oxidase of residual respiration of tissues in aseptic autolysis*

(The values were corrected for endogenous O_2 uptake of monoamine oxidase preparation.)

				With purified tyramine oxidase	
Animal	Organ	Time of autolysis (days)	O_2 (μl./hr.)	O_2 (μl./hr.)	Increase
Guinea pig	Liver	20	13·6	79·4	+65·39
	Liver	20	1·1	34·5	+33·46
	Kidney	20	1·2	20·8	+19·63
	Kidney	20	4·2	27·0	+22·84
Rat	Liver	25	6·7	64·9	+58·15
	Liver	25	2·7	38·9	+36·21
	Liver	26	0·9	88·1	+87·20
	Kidney	25	1·7	25·7	+23·97
	Kidney	26	1·0	27·5	+26·54
	Kidney	25	4·1	23·9	+19·79

Kohn showed that the activity of monoamine oxidase is increased 200–300 % in presence of high O_2 tensions, compared with activity in air. Thompson & Tickner were also able to confirm that the activity of the enzyme in air is only a third of the activity which occurs in presence of high O_2 tensions.

In the experiments reported above, good increases of activity were observed in 100 % O_2, but the degree increase was variable; in no experiment did the increase exceed 100 % and in a number of cases it was very much smaller. These facts suggest that, although monoamine oxidase is present in autolysing tissues, its functioning is nevertheless not optimal in these conditions.

Presence of amines in autolysing tissue homogenates

From the results of the preceding experiments the possibility arose that residual O_2 uptake by autolysing tissue homogenates was related to oxidation of amines present in these tissues. Amines could originate in the tissues by decarboxylation of amino-acids formed by catheptic hydrolysis of proteins.

Certain facts agreed with this hypothesis: (1) residual O_2 uptake is in general larger in the liver than in kidney; (2) residual O_2 uptake is smaller in the rat kidney than in other organs; (3) monoamine oxidase activity is greater in the liver than in kidney, being particularly low in rat kidney.

These points are in agreement with the findings of Pugh & Quastel (1937), Kohn (1937) and Sloane-Stanley (1949), who observed that monoamine oxidase and amino-acid decarboxylases are generally more abundant in the liver than in the kidney and are particularly scarce in rat kidney.

To verify this hypothesis, experiments were carried out to demonstrate the presence of free amines in autolysing tissue homogenates.

For these purposes an indirect criterion was employed, consisting of the measurement of extra O_2 uptake by the tissue homogenates when a purified monoamine oxidase preparation was added. O_2 uptakes, larger than the theoretical amounts (which result from the sum of endogenous O_2 uptake of the tissue homogenate plus the amount of O_2 consumed by the purified enzyme preparation), show the presence in the tissue homogenate of a substrate for added monoamine oxidase. Since the specificity of purified monoamine oxidase for amines has been demonstrated by Kohn (1937), it seemed likely that

Table 8. *Consumption of oxygen and production of ammonia by autolysing tissue homogenates in absence and presence of tyramine*

			Tyramine absent		Tyramine present		Atoms O_2 : mol. NH_3	
Animal	Organ	Time of autolysis (days)	O_2 consumed in 1 hr. (μl.)	NH_3 (μmol./hr.)	O_2 consumed in 1 hr. (μl.)	NH_3 (μmol./hr.)	Tyramine absent	Tyramine present
Guinea pig	Liver	20	25·1	1·08	42·7	2·06	2·06	1·83
	Liver	20	17·0	0·72	29·4	1·58	2·09	1·65
	Liver	22	27·3	1·32	59·5	3·62	1·83	1·43
	Kidney	20	21·5	0·98	38·9	2·74	1·94	1·25
Rat	Liver	25	17·7	0·80	40·1	2·42	1·96	1·47
	Kidney	25	6·4	0·30	12·0	0·82	1·88	1·30

a positive proof could be a valid argument for the presence of free amines in the autolysing tissue homogenates. The results of these experiments are reported in Table 7, and it is concluded that they provide evidence for the presence in autolysing tissue homogenates of a substrate for monoamine oxidase.

Consequently, it seems likely that the small amounts of O_2 which are taken up by these homogenates, even many days after the beginning of

Animal	Organ	Time of autolysis (days)
Rat	Liver	20

autolysis, are due to oxidation of amines present in the tissues by the surviving specific enzyme.

Presence of products of amine oxidation in autolysing tissue homogenates

Further studies were performed to show the possibility of occurrence in the autolysing tissues of products of oxidation of amines. According to Kohn, as a consequence of oxidation of amines, an aldehyde, hydrogen peroxide and ammonia are produced.

(*a*) *Production of ammonia* was first ascertained qualitatively with Nessler's reagent; afterwards, quantitative determinations were performed. Results are given in Table 8, from which it may be seen that ammonia is produced during residual respiration of autolysing tissues; NH_3 production increases remarkably in the presence of tyramine. In further experiments it was shown that an environment of 100 % O_2 can noticeably increase both O_2 uptake and ammonia production.

The calculation of the quotient 'atoms O_2 consumed : mol. NH_3 produced' shows that, in the absence of tyramine, the quantity of O_2 which is consumed is approximately double the theoretical amount: this fact could mean that only one half of O_2 uptake is related to the actual oxidation of free amines. The quotient is lower and nearer to the theoretical values in the experiments with tyramine.

(*b*) *Production of hydrogen peroxide* during residual respiration of the autolysing tissues was also studied. The results of experiments based on the observations of Keilin & Hartree (1936), involving the addition of 1 mg. of ethanol to the homogenates, show that ethanol is very slightly oxidized. One such experiment is summarized below:

O_2 (μl./hr.)	O_2 (μl./hr.) in presence of 1 mg. ethanol	Increase
19·5	23·1	+3·61

Since small extra O_2 uptakes observed in the presence of ethanol cannot be referred to the activity of ethanol dehydrogenase (on account of the inactivity of cytochrome oxidase in the autolysing tissues and of the presence of cyanide in the reaction mixture), it is likely that the phenomenon is a consequence of the oxidation of added ethanol by H_2O_2 which is produced in traces in the homogenates.

Also the second indirect criterion which was employed for the demonstration of production of H_2O_2 during residual respiration of autolysing tissues (oxidation of Hb to meta-Hb) gave positive results. At the end of the experimental period obvious changes had occurred in the colours of the fluids contained in the flasks; while the control solution remained a rose-red colour, the colour of the solution containing the filtrate of the tissue homogenate + Hb was brown.

(*c*) *Presence of aldehydes.* Extra O_2 uptake after addition to the homogenates of a purified xanthine oxidase (Schardinger's enzyme) was considered as proof of the presence of aldehydes in the homogenates.

Results of two experiments are reported below. (The values of O_2 uptake in the presence of the xanthine oxidase preparation were corrected for the endogenous O_2 uptake of the enzyme.)

Animal	Organ	Time of autolysis (days)	O_2 (μl./hr.)	O_2 (μl./hr.) in presence of xanthine oxidase preparation	Increase
Guinea pig	Liver	24	18·7	23·1	+4·38
	Kidney	24	13·3	18·5	+5·2

From these experiments it seems that addition of Schardinger's enzyme causes a small increase of O_2 uptake.

Additional experiments showed also that, when xanthine oxidase is added to the homogenates after 1 hr. of residual respiration rather than at the start of the experiment, O_2 uptake during the next half-hour shows slightly larger increases. The explanation of this may be that aldehydes are not pre-existing in the homogenates, but are produced during the period of respiration.

DISCUSSION

It is concluded from the experiments reported in this paper that a considerable amount of the residual O_2 uptake by homogenates of autolysing tissues is related to the oxidation by surviving monoamine oxidase of free amines which are present in these tissues.

Amines may be produced in autolysing tissues by catheptic hydrolysis of proteins and by decarboxylation of the amino-acids so produced. Catheptic hydrolysis of proteins can occur in the given experimental conditions (pH 4·5, absence of O_2); amino-acid decarboxylases are also active anaerobically. Monoamine oxidase, which shows optimal activity in weakly alkaline media and is dependent for its functioning upon the presence of O_2, cannot act, on the contrary, in the experimental conditions used. Accumulation of free amines could consequently occur.

This interpretation is in agreement with Kohn's finding, which was also confirmed in the present work, that in purified monoamine oxidase preparations kept in the cold store, endogenous O_2 uptake increases remarkably in the course of time, a likely consequence of liberation of substrate.

Monoamine oxidase itself undergoes some destruction in tissues which have been exposed for 20–30 days to autolysis, residual O_2 uptake and intensity of oxidation of tyramine decreasing after the first few days of autolysis; it is completely destroyed after 30–40 days.

From the experiments on ammonia production, it is concluded that the quotient 'atoms O_2 consumed: mol. NH_3 produced' is always greater than the theoretical, and it seems likely therefore that other oxidative processes, perhaps of minor importance, can occur during the residual respiration of the autolysing tissue homogenates.

Since conditions very similar to those used experimentally in the present work occur in ischemic necrosis of tissues, it seems possible that similar chemical processes may occur in ischemic necrotic tissues. This possibility may be of particular interest in connexion with the aetiology of certain hypertensive states.

SUMMARY

1. It has been confirmed that liver and kidney of guinea pigs, rats and mice undergoing aseptic autolysis can consume small quantities of oxygen even many days after the beginning of autolysis.

2. pH optimum for residual respiration of autolysing tissues occurs in a weak alkaline medium (pH 7·4–7·6); oxygen uptake increases noticeably in the presence of high oxygen tensions; cyanide and fluoride exert only a weak inhibitory action on the residual respiration, while *sec.*-octyl alcohol, 3 M-urea and 0·01 M-hydrazine hydrate inhibit strongly.

3. Succinoxidase, lactic dehydrogenase, α-glycerophosphate dehydrogenase, and D- and L-amino-acid oxidase are completely inactive in autolysing tissues.

4. Addition of tyramine or adrenaline hydrochlorides produces a marked increase of oxygen uptake.

5. Addition of purified monoamine oxidase to homogenates of autolysing tissues produces an increase of oxygen consumption, showing the presence of free amines.

6. Evidence is adduced for the production of ammonia and of traces of hydrogen peroxide and aldehyde compounds during residual respiration.

7. The hypothesis is presented that residual respiration of autolysing tissue homogenates is related to the oxidation of free amines present in the homogenates by a surviving monoamine oxidase.

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The Polarographic Estimation of Steroid Hormones

5. DETERMINATION OF PROGESTERONE IN BLOOD

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A reliable method for the determination of progesterone in blood would appear to have great value in both chemical and physiological fields. The common biological methods for the assay of this hormone are, with one exception, of relatively low sensitivity, requiring amounts of the order of 1 mg. for a definite response. In consequence, most of our knowledge of the metabolism of progesterone has been derived from studies of the excretion of the metabolite, pregnane-3α:20α-diol. Such studies suffer from the disadvantage that the urinary pregnanediol level will be influenced by liver and kidney function as well as by endogenous progesterone production. The value of a direct method which can be applied to blood is thus evident.

Haskins (1941) adapted the intrauterine injection technique of McGinty, Anderson & McCullough (1939) to the problem of the determination of progesterone in pregnancy blood. He used progestational changes in the uterus as end point, following the classical method of Corner & Allen (1929). He examined twenty pregnancy bloods and only in one case was able to detect progesterone, equivalent to 0·13 μg./ml. serum. This was at the bottom limit of sensitivity of the bioassay used. Hooker & Forbes (1947) developed a modified McGinty bioassay using the mouse as test animal and histological changes in the endometrial stromal nuclei as end point. The method was claimed to detect progesterone at a concentration of 0·3 μg./ml. Hooker & Forbes (1949*b*) later examined the specificity of the method. They found that, of the twenty-four steroids tested, progesterone alone gave the characteristic response. Application of the method to human pregnancy blood (Hooker & Forbes, 1949*a*) indicated a value of 5·3 μg./ml.; 90% of this was in the free state, the remaining 10% requiring hydrolysis to render it biologically active. Hooker & Forbes (1950) also applied the method to follow progesterone concentrations during the menstrual cycle of the monkey.

A chemical approach to the problem was made by Reynolds & Ginsburg (1942), who developed a method based on the ultraviolet absorption of extracts at 240 mμ. Haskins (1950) applied a similar method to pregnancy blood. In agreement with his earlier bioassay experiments and in contradiction to Hooker & Forbes (1949*a*), he was unable to detect any progesterone in the blood examined, although his method could detect 0·1 μg./ml. plasma. He also demonstrated that progesterone injected intravenously in the rabbit disappeared from the circulation very rapidly and that the disappearance rate was decreased by hepatectomy.

Our own interest in the problem was stimulated by the work of Hooker & Forbes (1949*a*). The levels found by them should be capable of polarographic determination as Girard complexes of Δ^4-3:20-diketones by the technique of Barnett, Henly & Morris (1946). A method has been developed based on extraction and partition between organic solvents, with final separation of the individual steroids by partition chromatography and subsequent polarographic estimation. A preliminary account of the work has appeared previously (Butt, Morris & Morris, 1949).

EXPERIMENTAL

Determination of progesterone in blood. Blood used in the procedure was collected in tubes containing heparin and worked up without delay.

Extraction and preliminary fractionation. The solvent partition method used is shown schematically in Fig. 1 and illustrated by the following example. Plasma (20 ml.) is poured slowly into 100 ml. of a 3:1 (v/v) ethanol-ethyl ether mixture with mechanical stirring. The mixture is stirred for a further 10 min., centrifuged and the supernatant liquid separated. The precipitate is washed twice by stirring with 50 ml. portions of the 3:1 ethanol-ethyl ether mixture. The combined extracts are concentrated *in vacuo* to a volume of 20 ml. and diluted with 40 ml. of water. The aqueous ethanol mixture is extracted three times with 60 ml. portions of ethyl acetate (A.R., redistilled). The ethyl acetate extracts are evaporated to dryness *in vacuo* at a bath temperature below 50°. The residue is dissolved in 70% aqueous methanol (A.R., redistilled) by warming, and transferred quantitatively to a 25 ml. flask, a total volume of 10 ml. being used. The mixture is stored for 18 hr. at −15°. The aqueous methanol is separated from the precipitated lipids by centrifugation in an angle centrifuge at 5000–6000 rev./min., the process being carried out in a refrigerator at −15°. The supernatant liquid is diluted with 20 ml. of water and extracted three times with 30 ml. portions of light petroleum (b.p. 40–60°; A.R., redistilled), The combined light petroleum extracts are washed twice with 30 ml. portions of water and evaporated to dryness *in vacuo* below 50°. The residue is

transferred quantitatively to a small tube with 1·0 ml. absolute ethanol, the ethanol removed *in vacuo* and the residue dissolved in 0·1 ml. 70% (v/v) methanol for transfer to the partition column. All solvents used in this procedure should be redistilled and a control experiment carried out to ensure that the polarographic blank is satisfactory, i.e. is indistinguishable from that of the base solution.

marked transverse layering. The column is finally washed with about 10 ml. of mobile phase. A column 6 × 0·6 cm. should flow under gravity alone at about 1 ml./hr. Excess mobile phase is removed from the top of the column with a capillary pipette and the liquid level forced down under 1–2 cm. Hg positive pressure until it is about 1 mm. below the surface of the Supercel. Sufficient dry Supercel is now

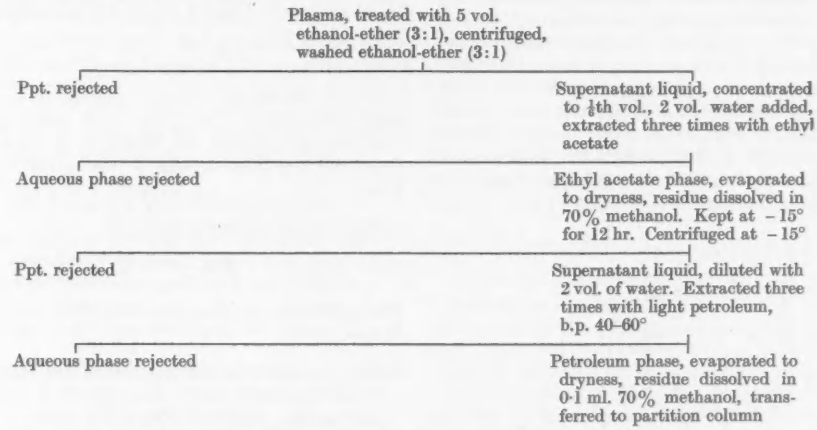
Plasma, treated with 5 vol. ethanol-ether (3:1), centrifuged, washed ethanol-ether (3:1)
Ppt. rejected
Supernatant liquid, concentrated to ⅛th vol., 2 vol. water added, extracted three times with ethyl acetate
Aqueous phase rejected
Ethyl acetate phase, evaporated to dryness, residue dissolved in 70% methanol. Kept at −15° for 12 hr. Centrifuged at −15°
Ppt. rejected
Supernatant liquid, diluted with 2 vol. of water. Extracted three times with light petroleum, b.p. 40–60°
Aqueous phase rejected
Petroleum phase, evaporated to dryness, residue dissolved in 0·1 ml. 70% methanol, transferred to partition column

Fig. 1. Preliminary partition system for separation of progesterone from blood.

Partition chromatography. Columns used have been 6 mm. in diameter and 6 cm. long. The glass tube should be about 15 cm. long, provided with a capillary outlet and a B14 standard joint at the upper end. For preparation of the column a Martin packer (Howard & Martin, 1950) is used. This consists of a stainless steel disk of diameter about 1 mm. less than that of the column. The disk has six to eight holes, 0·7 mm. in diameter and a long, central, stainless steel handle. Hyflo grade Supercel has been used as supporting phase throughout this work. Hyflo Supercel (50 g.) is treated with conc. HCl (200 ml.) and allowed to stand overnight. The mixture is heated at 100° for 1 hr., cooled and washed with water until the washings are neutral. The Supercel is finally air-dried at 110°. Aqueous methanol (70%) was used as stationary phase and *n*-hexane as mobile phase. The two phases should be very thoroughly equilibrated before use. Supercel (3 g.) is treated with 2 ml. stationary phase added in small portions with thorough mixing. The Supercel will still appear almost dry. Sufficient mobile phase is added to make a slurry. A small piece of absorbent cotton wool is forced down the column to the capillary outlet. A small portion of the Supercel slurry is transferred to the column with excess mobile phase and forced down with the Martin packer. This is moved rapidly up and down to give a uniform suspension. The packer is then moved down slowly to trap some of the Supercel under the disk and to pack it down firmly. The rest of the Supercel in suspension is then packed down in a similar manner. The operation is repeated by addition of further Supercel slurry to the column and continued until the required length is reached. Care should be taken that there is always some mobile phase above the Supercel and that no air bubbles are trapped during packing. A well packed column should appear quite uniform and not show any added to form a layer 1–2 mm. deep on the top of the column. The sample to be examined, dissolved in about 0·1 ml. stationary phase, is added to the dry Supercel, with a capillary pipette, and forced down under slight positive pressure. Mobile phase is now added carefully with a capillary pipette so that the top of the column is not disturbed. A separating funnel with standard joint, containing mobile phase, is next attached. This is provided with a capillary inlet so that it serves as a constant pressure vessel. The column is run and fractions collected as required.

The efficiency of packing may be tested on a 70% methanol-*n*-hexane column using Sudan III in *n*-hexane as test substance. The dead space in a 0·6 × 6 cm. column should be about 0·6 ml. (see p. 436).

Polarographic estimation. With the column dimensions given the first 1 ml. of eluate will contain no progesterone and is rejected. The second and third ml. of eluate, which contain the progesterone if present, are combined and evaporated to dryness *in vacuo* at a temperature not exceeding 50°. The residue is finally dried *in vacuo* over P_2O_5 overnight. The residue is treated with 0·02 ml. of a freshly prepared solution of Girard's reagent T (20 mg.) in anhydrous acetic acid (0·2 ml.) and heated for 2 min. at 100°. The reaction product is treated with 2 ml. of the polarographic base solution (0·5 N-NaCl, 1 vol.; 0·2 N-NaOH, 2 vol.; water, 1 vol.), transferred to the polarographic cell, and O_2 removed with a stream of N_2. General polarographic technique and purification of reagents follow the description of Barnett *et al.* (1946).

A standard curve is constructed using 5–20 μg. amounts of progesterone at sensitivity 4 μa. = 100 scale divisions and 2 μa. = 100 scale divisions of the Tinsley polarograph.

RESULTS

Separation of individual steroids on partition columns

The separation of progesterone, androst-4-ene-3:17-dione, deoxycorticosterone and testosterone with different compositions of stationary phase is shown in Fig. 2. As a result of these experiments 70% aqueous methanol was chosen as stationary phase as it permits of a clear separation of progesterone from androst-4-ene-3:17-dione, which appears to behave most like progesterone among the naturally occurring Δ^4-3-ketosteroids. The symmetry of the elution curves suggests that the separation mechanism is mainly partition. This was tested by comparison of the partition coefficients calculated from the elution curves with those measured directly.

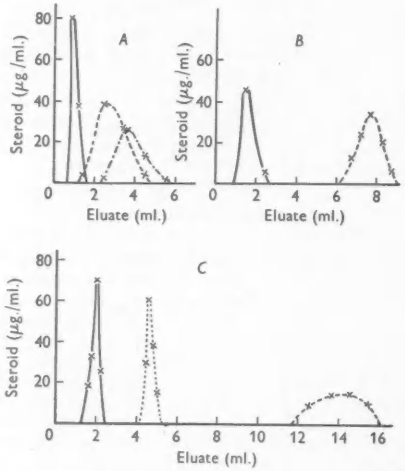

Fig. 2. Separation of steroids on partition columns. *A*, 40% methanol-*n*-hexane column; *B*, 60% methanol-*n*-hexane column; *C*, 70% methanol-*n*-hexane column. Progesterone, ——; androst-4-ene-3:17-dione, ...; deoxycorticosterone, – – –; testosterone, –·–·–.

The partition coefficients were calculated from the elution curve maxima by a modification of the equation of Martin & Synge (1941). If A = cross-sectional area of the column (sq.cm.), A_S = cross-sectional area of the stationary phase (sq.cm.), A_L = cross-sectional area of the mobile phase (sq.cm.), L = length of column (cm.), K = partition coefficient = (concentration of solute in stationary phase)/(concentration of solute in mobile phase), R = (rate of band movement on column)/(rate of liquid surface movement above column), then

$$K = \frac{A}{RA_S} - \frac{AL}{A_S}. \quad (1)$$

If V_r = the retention volume = volume of eluate to peak concentration (ml.), the band maximum will have moved through the length L of the column when the retention volume V_r has passed through. Thus

$$R = AL/V_r. \quad (2)$$

Substituting for R in equation (1)

$$K = \frac{V_r}{LA_S} - \frac{A_L}{A_S}. \quad (3)$$

The values of V_r for the steroids studied are given in Table 1.

Table 1. *Retention volumes of steroid ketones on Supercel columns with* n*-hexane as the mobile phase and aqueous methanol as the static phase*

Steroid	Concn. of methanol (%)	Retention volume (ml.)
Progesterone	40	0·94
	60	1·6
	70	2·0
	80	3·5
Androst-4-ene-3:17-dione	70	4·8
Deoxycorticosterone	40	2·5
	60	7·7
	70	14·4
Testosterone	40	3·6

A_S was determined from the dimensions of the column and the volume of stationary phase used. A_L was determined from the column 'dead volume', which is equal to the retention volume of a solute of zero partition coefficient. The dye Sudan III was used for this purpose; 'dead volume' determinations were carried out on the columns immediately after the steroid experiments. Values were from 0·65 to 0·70 ml.

Table 2. *Comparison between partition coefficients measured directly and calculated from column data*

(See Eqn. 3 and Table 1. Mobile phase *n*-hexane.)

Substance	Stationary phase (% methanol)	K (concn. stationary phase/concn. mobile phase) Calc.	Direct
Progesterone	40	0·39	0·42
	70	1·95	1·95
Deoxycorticosterone	40	2·76	2·66
	70	19·6	23·5
Androst-4-ene-3:17-dione	70	6·0	5·6

Partition coefficients were determined directly by equilibrating the steroid between the two phases and determining the concentrations in both phases polarographically. Comparisons of the two values for K for some steroids with different phase concentrations are given in Table 2. The agreement over a wide range of K values indicates that the column mechanism is essentially partition.

The number of theoretical plates in the columns used were calculated for some of the elution curves using the equation of Matheson (1949). Values of sixty to eighty theoretical plates for a 6 cm. column were obtained. The height equivalent to a theoretical plate was thus rather less than 1 mm.

Recovery of progesterone from plasma

Some typical recoveries of progesterone added to 20 ml. samples of plasma, which was then taken through the routine process, are given in Table 3. It will be seen that even at the 10 μg. level recoveries are satisfactory. 1 μg. can easily be detected.

Table 3. *Recovery of progesterone added to plasma*

(20 ml. plasma used.)

Progesterone added (μg.)	Recovery (%)
10	85
10	91
10	89
20	94
20	89
20	86
30	90
30	96
30	92

Recovery of progesterone from circulating blood

Normal rats. In order to test the method more rigorously and to obtain an estimate of the rate of disappearance of progesterone from the circulation, progesterone (1–3 mg. in 0·4 ml. 50% aqueous ethanol) was injected intravenously into normal rats and blood samples for analysis taken 5 min. later. Results are given in Table 4. It will be seen that more than 95% of the injected progesterone has been removed from the circulation in 5 min.

Rats with ligated livers. Since Haskins (1950) has shown that hepatectomized rabbits eliminate progesterone from the circulation rather more slowly than normal animals, it was considered of interest to carry out similar experiments in the rat. The hepatic vein and artery were clamped and the animals left 10 days for shunting anastomoses to develop. The hepatic vein and artery were finally ligated before injection. This procedure minimizes trauma and formation of blood depots. The results are shown in Table 4. The recoveries obtained are only slightly higher than those obtained with normal animals.

Plasma progesterone in pregnancy

Blood samples of 12–20 ml. from thirteen women with pregnancies from 12 weeks to full term, were examined by the routine method. In no case was any progesterone detected. In one case the plasma extract was subjected to acid hydrolysis by the method of Hooker & Forbes (1949*a*) prior to the routine fractionation process. In this case also, no progesterone was detected. Blood from a woman suffering from infective hepatitis and with a pregnancy of 16 weeks' duration was also examined. A blood level of 0·4 μg. progesterone/ml. of blood was found here.

Progesterone in human placental blood

The routine method was also applied to human placental blood obtained from the umbilical cord. The results are given in Table 5. In two cases progesterone was detected, and in two further cases (nos. 3 and 4) a substance giving a polarographic wave resembling that characteristic of Δ^4-3-ketosteroids but showing no 20-ketosteroid wave was detected. The significance of this cannot at present be evaluated.

Table 4. *Rate of elimination of progesterone from the circulation of rats with normal and impaired liver function*

(Recoveries calculated on a blood volume of 6 ml./100 g. body weight.)

Rat no.	Body wt. (g.)	Progesterone injected (mg.)	Sampling time (min.)	Recovery (%)
		Normal rats		
1	190	1	5	1·4
2	195	2	5	4·7
3	200	3	5	2·4
4	200	1	9	0·0
		Rats with ligated livers		
1	240	3	5	3·7
2	270	3	5	3·2
3	280	3	5	3·9

Table 5. *Estimation of progesterone in placental blood*

No.	Sex of infant	Progesterone (μg./ml.)	Remarks
1	F	1·3	Normal } biovular
2	F	0·3	Small } twins
3	M	0	Normal
4	M	0	Normal
5	F	0	Arterial blood only

DISCUSSION

The results of the present work show that the amounts of progesterone present in blood obtained from cases of pregnancy of varying duration were in all cases below 0·1 μg./ml., the limit of sensitivity of the method with sample volumes of 20 ml. of plasma. This is in agreement with the findings of Haskins, using both biological and physico-chemical methods. Dr K. Dobriner (Sloan-Kettering Institute, New York) has also informed the authors privately that he was unable to isolate any progesterone from several litres of pregnancy blood using the methods of fractionation and infrared spectroscopy developed in his laboratory. These findings are in conflict with those of Hooker & Forbes (1949*a*) obtained by their intrauterine injection bioassay technique. It thus appears likely that the substance responsible for the high plasma 'progesterone' values of the Hooker & Forbes method is not in fact progesterone, although the possibility of an augmentation effect in the bioassay produced by some unknown factor in blood in conjunction with plasma progesterone levels below 0·1 μg./ml. cannot be excluded.

The adequacy of the present technique under physiological conditions appears to be substantiated by the detection of progesterone in the hepatitis case and in placental blood as well as by the rat experiments.

The value of the partition column method for the quantitative separation of steroids is well illustrated by this work. The chief advantages appear to be: (1) symmetrical elution curves at the concentration used, obviating losses through 'tailing'; (2) independence of the properties of the solid phase, in marked contrast to adsorption chromatography; (3) simple calculation of retention volumes from partition data, and of column dimensions necessary for a given separation; (4) the possibility of continuous variation of column behaviour by alteration of the stationary phase.

SUMMARY

1. A method for the determination of progesterone in blood, based on solvent partition, partition chromatography and polarographic determination as a 3:20-ketosteroid, is described.

2. The concentration of progesterone in the circulation after injection into normal and partially hepatectomized rats has been examined.

3. Application of the method to human pregnancy blood has shown that the progesterone level is less than 0·1 μg./ml.

4. Progesterone has been detected in human placental blood.

The authors are indebted to Miss M. L. Dedman for assistance with the animal experiments, to Messrs Organon Laboratories for a gift of progesterone, and to Messrs British Schering Laboratories for a gift of deoxycorticosterone.

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Some Aspects of the Metabolism of D- and L-Serine by the Rat

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In a previous paper (Arnstein, 1951) L-serine was shown to be a precursor of both the ethanolamine moiety and the methyl groups of choline. It was found that the *in vivo* conversion of L-serine to ethanolamine takes place by loss of the carboxyl group and that the β-carbon atom is incorporated into choline methyl groups, presumably via a one-carbon fragment. Recently, the biosynthesis of choline methyl groups from one-carbon sources has also been reported from several other laboratories (du Vigneaud, Verly & Wilson, 1950; Elwyn & Sprinson, 1950*a*; Sakami & Welch, 1950; Jonsson & Mosher, 1950).

The conversion of serine to choline is stereochemically specific, as D-serine did not give rise to either the ethanolamine moiety or the methyl groups of choline (Arnstein, 1951). It was of interest, therefore, to compare the D- and L-isomers of serine in other biological reactions, and this paper deals with the conversion of both isomers, labelled with ^{14}C in the β-position, into acetate and glycine. Recently, Elwyn & Sprinson (1950*b*) showed that only the L-isomer of serine is converted to uric acid, the β-carbon atom giving rise to carbon atoms 2 and 8 of uric acid.

EXPERIMENTAL

Feeding experiments and isolation of metabolites

Animals and diet. The animals used in these experiments were either albino rats of Institute stock or Wistar strain rats supplied by Dr K. Coward, Pharmaceutical Society (P.S. strain).

The animals were kept on Institute stock diet previously described (Arnstein, 1951). The isotopically labelled compounds were fed overnight mixed with diet containing DL-α-amino-γ-phenylbutyric acid and sodium benzoate or *p*-aminobenzoic acid (see Table 1) and the overnight urine was collected.

Isolation of hippuric acid and acetamido-acids from urine. Hippuric acid or *p*-acetamidobenzoic acid (ABA) was separated from L-α-acetamido-γ-phenylbutyric acid (APBA) by a four-stage counter-current extraction (Bush & Densen, 1948) of the acidified urine with $CHCl_3$ and ethyl acetate (Arnstein & Neuberger, 1949). The urine was extracted four times with $CHCl_3$ (3–4 vol.), each $CHCl_3$ layer being subjected to counter-current extraction with water (1 vol.). The aqueous layers were then combined and extracted in a similar fashion with ethyl acetate. The organic solvents were dried and evaporated, hippuric acid or ABA being obtained from the ethyl acetate and APBA from the $CHCl_3$ solution. The specific radioactivity of hippuric acid, ABA or APBA obtained by this method and crystallized once from water was unchanged after several recrystallizations.

Isolation of xanthydryl urea from urine. A solution of xanthydrol in methanol (10% w/v; 2 ml.) was added to 3–4 drops of urine dissolved in a mixture of acetic acid (7 ml.) and water (2 ml.). Xanthydryl urea crystallized almost immediately. The product was washed once with acetic acid-water (7:2, v/v) and twice with ether, and dried at 80°.

Location of radioactivity in glycine and acetic acid. The hippuric acid was hydrolysed by refluxing overnight with 6N-HCl. The solution was extracted with ether to remove benzoic acid and evaporated to dryness *in vacuo*. Potassium phosphate buffer (3·5 g. K_3PO_4 + 20 g. KH_2PO_4/100 ml. water; pH, 5·5) (10–20 ml.) was then added and the glycine was degraded by reaction with ninhydrin at 100°. The formaldehyde was steam-distilled from the reaction mixture and isolated as the dimedone derivative; at the same time the CO_2 arising from the carboxyl group of glycine was collected as $BaCO_3$.

The acetamido acids were hydrolysed by refluxing for several hours with 2N-H_2SO_4. Acetic acid was separated by steam distillation and converted to the lithium salt, preferably by titration with Li dissolved in methanol in order to avoid contamination with carbonate. Lithium acetate was degraded by pyrolysis of the dry salt at 400° *in vacuo*, the acetone thus formed being condensed in a liquid-air trap and further degraded to iodoform. Alternatively, lithium acetate was converted to silver acetate, which was then decarboxylated by reaction with Br_2 in dry CCl_4 (Hunsdiecker & Hunsdiecker, 1942).

Isolation and degradation of serine from urine after feeding D-[β-^{14}C]-*serine.* D-[β-^{14}C]-Serine (0·5252 g.; specific radioactivity, 501 μc./mol.) mixed with stock diet (5 g.) containing D-glucose (0·5 g.) was fed to four adult female rats of Institute strain (aggregate body weight 1070 g.). The overnight urine was collected (volume of urine and washings, 63 ml.) and chromatographed directly on Zeo-Karb 225 ion-exchange resin (Permutit Co., Ltd.; 20 g.; dimensions of column: 20 × 1·4 cm. diameter). The amino-acids were eluted with 0·15M-aqueous NH_3, approximately 5 ml. fractions being collected. Selected fractions were analysed by paper chromatography and the main serine-containing fractions (fractions 80–100) were combined and evaporated to dryness (yield of crude solid, 280 mg.). The crude serine was converted to the *p*-hydroxyazobenzenesulphonate, which was recrystallized from water (specific activity, 396 μc./mol.). The specific activity of this material was raised slightly by recrystallization, but as insufficient material for repeated crystallizations was available, it was converted to free serine and oxidized with periodate (Rees, 1946). The formaldehyde was isolated as the dimedone derivative

Table 1. *Details of administration of isotopically labelled compounds*

Exp. no.	Details of animals: No. of rats	Sex	Aggregate body wt. (g.)	Strain*	Details of labelled compounds fed: Compound	Wt. (mg.)	Radioactivity (μc.)	Specific radioactivity (μc./mmol.)	Quantity fed (mmol./100 g. body wt.)	Diet: Amount of food eaten (g.)	Compounds added/g. diet
1	2	F	600	I.S.	DL-$^{14}CH_2OH.CHNH_2.CO_2H$	160·2	4·0	2·62	0·254	20·0	{20 mg. α-amino-γ-phenylbutyric acid 20 mg. *p*-aminobenzoic acid
2	1	F	300	I.S.	DL-$^{14}CH_2OH.CHNH_2.CO_2H$	80·0	2·0	2·62	0·254	10·0	{20 mg. α-amino-γ-phenylbutyric acid 20 mg. sodium benzoate
3	2	F	550	P.S.	L-$^{14}CH_2OH.CHNH_2.CO_2H$	122·0	10·0	8·60	0·211	10·0	{20 mg. α-amino-γ-phenylbutyric acid 20 mg. sodium benzoate
4	2	F	508	P.S.	D-$^{14}CH_2OH.CHNH_2.CO_2H$	142·6	11·7	8·59	0·267	20·0	{20 mg. α-amino-γ-phenylbutyric acid 20 mg. sodium benzoate
5	2	M	637	I.S.	D-$^{14}CH_2OH.CHNH_2.CO_2H$	81·3	6·67	8·59	0·122	15·5	{20 mg. α-amino-γ-phenylbutyric acid 20 mg. *p*-aminobenzoic acid
6	2	M	692	I.S.	L-$^{14}CH_2OH.CHNH_2.CO_2H$	100·5	8·25	8·60	0·138	20·0	{20 mg. α-amino-γ-phenylbutyric acid 20 mg. *p*-aminobenzoic acid
7	1	F	302	P.S.	$^{14}CH_2NH_2.CO_2H$	9·6	9·6	75·07	0·0423	10·0	{20 mg. α-amino-γ-phenylbutyric acid 20 mg. *p*-aminobenzoic acid
8	2	F	542	I.S.	$^{14}CH_3CO_2Na$	3·07	37·5	1000	0·0275	16·0	{25 mg. α-amino-γ-phenylbutyric acid 18·8 mg. sodium benzoate

* Institute strain (I.S.) or Pharmaceutical Society strain (P.S.).

(m.p. 189°, after recrystallization from aqueous ethanol) and counted after dilution with carrier. The calculated specific radioactivity of the undiluted formaldehyde dimedone derivative was 450 μc./mol.

Synthesis of labelled compounds used in feeding experiments

All melting points are uncorrected.

Sodium [^{14}COOH] *acetate.* This compound was supplied by the Radiochemical Centre, Amersham.

[α-^{14}C]-*Glycine.* [2-^{14}C]-Bromoacetic acid, supplied by the Radiochemical Centre, Amersham, was treated with excess aqueous NH_3 (sp.gr. 0·88), essentially as described by Olsen, Hemingway & Nier (1943).

D- *and* L-[β-^{14}C]-*Serine.* Serine labelled in the β-position was prepared by the condensation of ethyl *p*-nitrobenzamidomalonate (I) with [^{14}C]-formaldehyde. ^{14}C-*p*-Nitrobenzoyl-DL-[β-^{14}C]-serine (III), obtained by partial hydrolysis of the condensation product (II), was resolved by preparing the quinine and brucine salts (Fischer & Jacobs, 1906) and hydrolysed to give both optical isomers of serine (Arnstein, 1949).

In the modification of King's synthesis of serine (King, 1947) (p. 441) pyridine was found to be a more convenient catalyst than NaOH for the condensation of formaldehyde with acylamidomalonic esters. An attempt to introduce the isotope at a later stage of the synthesis by condensing [^{14}C]-formaldehyde with the sodium salt of ethyl hydrogen *p*-nitrobenzamidomalonate was not successful, although the sodium salt of ethyl hydrogen acetamidomalonate was found to react as readily as the diester.

Other syntheses of serine labelled with ^{14}C in the β-position have recently been reported (Levine & Tarver, 1950; Elwyn & Sprinson, 1950*b*).

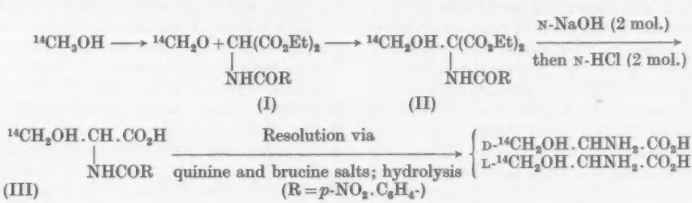
Condensation of formaldehyde with diethyl acetamidomalonate, using pyridine as catalyst. Diethyl acetamidomalonate (54·25 g.), aqueous formaldehyde (38·8% (w/w); 21 g.) and pyridine (1 ml.) were shaken together for 20 hr. The homogeneous solution was evaporated to dryness *in vacuo* and dried by azeotropic distillation with benzene. After further drying *in vacuo* crystalline diethyl hydroxymethylacetamidomalonate (60 g., 97%), m.p. 61–66°, was obtained (cf. King, 1947).

The O-*acetyl derivative* was prepared by heating the above diethyl hydroxymethylacetamidomalonate (25 g.) with acetic anhydride (20 ml.) and anhydrous sodium acetate (10 g.) for 1·5 hr. on the steam bath. Water (50 ml.) was then added and after 2 days at 0° the solution was extracted with ether. The ether extract was washed with aqueous $NaHCO_3$ and water, dried (Na_2SO_4) and evaporated to dryness. The residue was distilled, yielding a colourless, viscous oil, b.p. 140°/0·005 mm. Hg, which crystallized on standing (yield, 18 g.; m.p. 50°). (Found: C, 49·9; H, 6·5; N, 4·8. $C_{12}H_{19}O_7N$ requires C, 49·8; H, 6·6; N, 4·9%.)

Ethyl hydrogen acetamidomalonate. 1·01 N-NaOH (45·5 ml.) was added to a hot solution of diethyl acetamidomalonate (10 g.) in 50% (v/v) aqueous ethanol (50 ml.). After cooling, 2 N-H_2SO_4 (25 ml.) was added. The solution was saturated with NaCl and extracted twice with an equal volume of ethyl acetate. The extract was washed with a little water, dried (Na_2SO_4) and evaporated *in vacuo*, giving a crystalline residue (7·3 g., 84%), m.p. 129–130°. Two recrystallizations from water afforded the pure *compound* (prisms), m.p. 131°

(decomp. with evolution of gas). (Found: C, 44·8; H, 5·6; N, 6·9, 7·3. $C_7H_{11}O_5N$ requires C, 44·4; H, 5·9; N, 7·4%.)

Condensation of formaldehyde with the sodium salt of ethyl hydrogen acetamidomalonate. To a solution of the above half-ester (1·89 g.) in N-NaOH (9·9 ml.) was added a slight excess of a dilute aqueous solution of formaldehyde (16·6 ml.; giving *diethyl* p-*nitrobenzamidomalonate* (40 g.), m.p. 135–138°; a further crop (15 g.), m.p. 132–135°, was obtained by adding ether to the mother liquors. Two recrystallizations from $CHCl_3$ and benzene respectively afforded the pure compound (needles), m.p. 136°. (Found: C, 52·0; H, 4·8; N, 8·6. $C_{14}H_{16}O_7N_2$ requires C, 51·9; H, 5·0; N, 8·6%.)



20·6 mg./ml.) and pyridine (0·05 ml.). After 4 days at room temperature the solution was evaporated almost to dryness on the steam bath in a stream of air. 1·05 N-HCl (9·5 ml.) was added to the warm solution, when there was an immediate evolution of CO_2. After 0·5 hr. on the steam bath, 10 N-HCl (5 ml.) was added and the mixture was refluxed for 1·5 hr. The solution was evaporated to dryness, a little water was added and the evaporation repeated. After drying *in vacuo* over KOH, the residue was extracted with 95% ethanol. The solution was filtered and treated with a slight excess of pyridine; after cooling, crystalline serine (0·73 g., 70%) was obtained.

[^{14}C]-*Formaldehyde.* [^{14}C]-Methanol (32·5 mg., containing approximately 1 mc. of ^{14}C), obtained from the Radiochemical Centre, Amersham, was diluted with non-radioactive methanol (378·9 mg.) and oxidized to formaldehyde in the apparatus, made of Pyrex tubing, shown in Fig. 1. The vessel (*A*), containing the methanol, was immersed in a bath kept at 20° and dry air free from CO_2 was admitted through the inlet (*B*) at a rate of 120–150 ml./min. With this rate of flow and temperature, vaporization of the methanol occurred at the required rate, giving a methanol:air ratio of between 1:10 and 1:15 (v/v). When desired, additional air or O_2 could be admitted at *B'*, but this was found to be unnecessary.

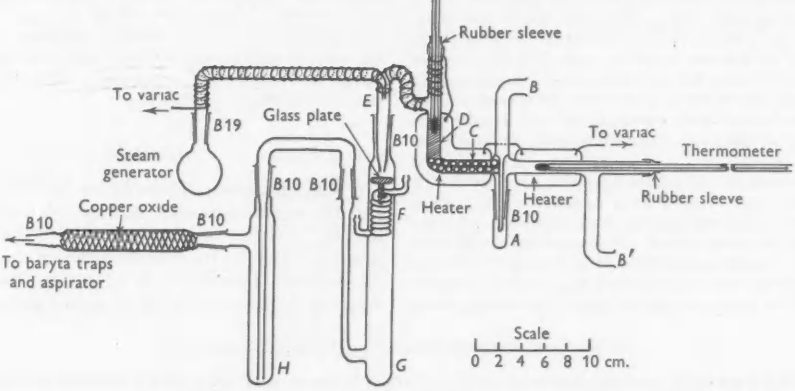


Fig. 1. Apparatus for preparation of [^{14}C]-formaldehyde. For details of components, see text. *B*10 and *B*19 are standard ground joints.

Diethyl p-*nitrobenzamidomalonate.* Finely powdered p-nitrobenzoyl chloride (46·7 g.) was added in small portions to a stirred mixture of diethyl amidomalonate (44·5 g.) and pyridine (40·6 ml.) cooled in ice. Crystals began to separate almost immediately from the warm solution. After cooling, sufficient $CHCl_3$ to dissolve the solid was added. The $CHCl_3$ solution was washed with aqueous $NaHCO_3$ and water, and dried (Na_2SO_4). The solvent was evaporated *in vacuo*,

Thorough mixing and preheating of the reaction mixture was accomplished by passing the gases through a length of heated tube (*C*) packed with glass beads (4 mm. diameter). The heaters were constructed with 'Nichrome' resistance wire, which was wound on to the glass tubes covered with thin moistened asbestos sheets. After drying, the wire was held in place by the asbestos, which also served as insulating material. The temperature of the heaters was controlled by a

Variac transformer. The catalyst, consisting of molybdenum oxide (90%)-iron oxide (10%) mixture (Meharg & Adkins, 1933) in the form of moderately coarse lumps, was packed into the vertical part of the tube (*D*), which was heated to 350–400°. Condensation of polymer was prevented by warming the exit vapours and by injection of steam at *E*. After cooling by the internal spiral water condenser (*F*), the aqueous solution of formaldehyde was frozen in the liquid-air trap (*G*). A second trap (*H*), cooled in ethanol-solid CO_2, condensed only traces of radioactive material, presumably formaldehyde or unreacted methanol. The exit gases, containing CO, were passed through a heated tube filled with CuO, and the CO_2 formed was collected in two baryta traps (not shown in Fig. 1). The [^{14}C]-formaldehyde was estimated by titration with I_2 in alkaline solution (Fresenius & Grünhut, 1905) and counted as the dimedone derivative. Yield of aqueous formaldehyde solution: 294 mg. CH_2O (0·545 mc.)/10 ml. $BaCO_3$ recovered: 273·5 mg. (0·050 mc.).

Condensation of [^{14}C]-*formaldehyde with diethyl* p-*nitrobenzamidomalonate.* The solution of [^{14}C]-formaldehyde (9·6 ml., 0·524 mc.) was condensed with a slight excess of diethyl *p*-nitrobenzamidomalonate (3·25 g.) in 50% (v/v) aqueous pyridine (30 ml.), as described above. Hydrolysis of the intermediate, which was not isolated, gave crude *N*-*p*-nitrobenzoyl-DL-[β-^{14}C]-serine, m.p. 167–173° (1·80 g., 0·269 mc.; 51·5%). This material was diluted with carrier *p*-nitrobenzoyl-DL-serine (5·70 g., m.p. 205°) and recrystallized from water (yield, 6·15 g., 0·217 mc.).

D-[β-^{14}C]-*Serine.* The diluted *p*-nitrobenzoyl-DL-[β-^{14}C]-serine (6·15 g.) and quinine (7·85 g.) were dissolved in 50% (v/v) aqueous ethanol (273 ml.). After 18 hr. at 0° the crystalline quinine salt was filtered off; yield, after drying *in vacuo* over P_2O_5, 7·64 g. After two recrystallizations from 50% (v/v) aqueous ethanol (62·5 ml.), the *p*-nitrobenzoyl-[β-^{14}C]-D-serine quinine salt (5·89 g., 84%) was dissolved in warm 50% aqueous ethanol (56 ml.), N-NaOH (10·03 ml.) was added and the solution cooled at once. The mixture was cooled in ice for about 0·5 hr. and decanted. The supernatant solution was evaporated *in vacuo* to remove the ethanol, the residue was shaken with water (20 ml.) and filtered. The filtrate was acidified with 10 N-HCl, when the crude *p*-nitrobenzoyl-D-[β-^{14}C]-serine (1·97 g., 64·1%) crystallized. The crude product was refluxed for 5 hr. with 5 N-HCl (50 ml.). The solution was evaporated to dryness *in vacuo*, water (20 ml.) was added and the evaporation repeated. After the addition of hot water (50 ml.) the solution was cooled overnight at 0°, filtered and evaporated to dryness. The residue was dissolved in aqueous ethanol (95%, v/v; 20 ml.), treated with pyridine (2 ml.) in ethanol (5 ml.), and cooled, giving crystalline D-[β-^{14}C]-serine (0·62 g., 48·3%). After one recrystallization from water-ethanol the D-serine had a specific radioactivity 8·59 μc./mmol. and $[\alpha]_D^{23°} = +6·60° \pm 0·2°$, $c = 10·66$ in water, 1 dm. tube. This material was diluted with non-radioactive D-serine and oxidized with periodate (Rees, 1946); the radioactivity was located entirely in the β-position, within the limits of the counting error ($\pm 1·5$%).

L-[β-^{14}C]-*Serine.* The mother liquors from the preparation of *p*-nitrobenzoyl-D-[β-^{14}C]-serine quinine salt were evaporated *in vacuo* to remove the ethanol. The residue was dissolved in warm water (110 ml.) and N-NaOH (13·35 ml.) was added, the solution being cooled at once in ice. After several hours at 0° the solution was filtered, concentrated to about 10 ml. and acidified with 5 N-HCl (5 ml.). The crude *p*-nitrobenzoyl-L-[β-^{14}C]-serine (2·54 g., 83%) and brucine (3·96 g.) were dissolved in water (20·3 ml.). On cooling, the brucine salt separated and was recrystallized twice from water (12·5 ml.). The brucine salt (4·53 g.) was then dissolved in hot water (40 ml.), 1·01 N-NaOH (7·08 ml.) was added and the solution was cooled at once in ice. A further quantity of water (20 ml.) was added and after 18 hr. at 0° the crystalline precipitate was filtered off and washed with water (100 ml.). The filtrate was extracted three times with ether (100 ml.), acidified to litmus with 2 N-HCl (1 ml.) and concentrated *in vacuo* to approximately 5 ml. at a bath temperature of 60°. The solution was then acidified to congo red with 2 N-HCl, when *p*-nitrobenzoyl-L-[β-^{14}C]-serine (1·59 g., 51·7%) was obtained. The crude product was refluxed for 5 hr. with 3 N-HCl (40 ml.). The solution was cooled, filtered and evaporated to dryness *in vacuo*. The residue was dissolved in 95% ethanol (20 ml.), treated with pyridine (1 ml.) in ethanol (10 ml.) and cooled, giving crystalline L-[β-^{14}C]-serine (0·413 g., 32·5%). After one recrystallization from water-ethanol the L-serine had a specific radioactivity 8·60 μc./mmol.; $[\alpha]_D^{20°} = -6·59° \pm 0·4°$, $c = 7·685$ in water, 1 dm. tube. This material was diluted with non-radioactive DL-serine and oxidized with periodate (Rees, 1946); the radioactivity was located entirely in the β-position, within the limits of the counting error ($\pm 1·5$%).

RESULTS

The specific radioactivities of the xanthydryl urea and the acylamino-acids isolated from urine after feeding both stereoisomers of [β-^{14}C]-serine are given in Table 2. In order to compare the different experiments the dilutions of isotope have been calculated (Table 3) and are expressed as the relative

Table 2. *Specific radioactivities of isolated compounds*

(The radioactivity measurements were carried out with a Geiger counter, using infinite thickness samples.)

Exp. no.	Labelled compound fed	Specific radioactivity of isolated compound (μc./mol.) Xanthydryl urea	Hippuric acid	*p*-Acetamidobenzoic acid	L-α-Acetamido-γ-phenylbutyric acid
1	DL-$^{14}CH_2OH.CHNH_2.CO_2H$	—	—	5·74	19·4
2	DL-$^{14}CH_2OH.CHNH_2.CO_2H$	—	10·0	—	16·7
3	L-$^{14}CH_2OH.CHNH_2.CO_2H$	5·26	2·95	—	4·32
4	D-$^{14}CH_2OH.CHNH_2.CO_2H$	9·46	4·74	—	10·0
5	D-$^{14}CH_2OH.CHNH_2.CO_2H$	2·69	—	20·02	81·38
6	L-$^{14}CH_2OH.CHNH_2.CO_2H$	3·74	—	4·85	7·26
7	$^{14}CH_2NH_2.CO_2H$	6·07	—	20·24	9·92
8	$^{14}CH_3CO_2Na$	—	14·1	—	376·0

Table 3. *Dilution of isotopic tracer in isolated compounds*

(The dilutions of tracer are expressed as the relative isotope coefficient (RIC) and the concentration coefficient (CC), as defined by Anker, 1948.)

		Compounds isolated							
		Xanthydryl urea		Hippuric acid		*p*-Acetamido-benzoic acid		L-α-Acetamido-γ-phenylbutyric acid	
Exp. no.	Labelled compound fed	RIC ($\times 10^{-3}$)	CC ($\times 10^{-3}$)	RIC ($\times 10^{-3}$)	CC ($\times 10^{-3}$)	RIC ($\times 10^{-3}$)	CC ($\times 10^{-3}$)	RIC ($\times 10^{-3}$)	CC ($\times 10^{-3}$)
1, 2	DL-$^{14}CH_2OH.CHNH_2.CO_2H$	—	—	3·82	29·4	2·19	17·2	7·05	53·0
3	L-$^{14}CH_2OH.CHNH_2.CO_2H$	0·612	2·89	0·343	1·62	—	—	0·502	2·38
4	D-$^{14}CH_2.OH.CHNH_2.CO_2H$	1·10	4·11	0·552	2·06	—	—	1·16	4·35
5	D-$^{14}CH_2OH.CHNH_2.CO_2H$	0·313	2·57	—	—	2·33	19·1	9·48	77·6
6	L-$^{14}CH_2OH.CHNH_2.CO_2H$	0·435	3·15	—	—	0·564	4·09	0·844	6·12
7	$^{14}CH_2NH_2.CO_2H$	0·081	1·91	—	—	0·270	6·34	0·132	3·11

Table 4. *Dilution factors of isotopic tracer in isolated compounds*

(The dilution factor is the reciprocal of the concentration coefficient as defined by Anker (1948). It is the dilution of isotope which would have been observed if exactly 1 mmol./100 g. body wt. had been fed; Arnstein, 1951.)

			Dilution factor			
Exp. no.	Strain of rat*	Labelled compound fed	Urea	Glycine (of hippuric acid)	*p*-Acetamido-benzoic acid	L-α-Acetamido-γ-phenyl-butyric acid
1, 2	I.S.	DL-$^{14}CH_2OH.CHNH_2.CO_2H$	—	34·0	58·2	18·9
3	P.S.	L-$^{14}CH_2OH.CHNH_2.CO_2H$	346	618	—	420
4	P.S.	D-$^{14}CH_2OH.CHNH_2.CO_2H$	243	485	—	230
5	I.S.	D-$^{14}CH_2OH.CHNH_2.CO_2H$	389	—	52·4	12·9
6	I.S.	L-$^{14}CH_2OH.CHNH_2.CO_2H$	318	—	245	163·5
7	P.S.	$^{14}CH_2NH_2.CO_2H$	524	—	158	322

* Institute strain (I.S.) or Pharmaceutical Society strain (P.S.).

Table 5. *Distribution of isotopic tracer in the acetyl groups of excreted acetamido-acids*

			Distribution of radioactivity in acetate		
Exp. no.	Labelled compound fed	Compound isolated	Methyl carbon atom (%)	Carboxyl carbon atom (%)	Degradation method
1, 2	DL-$^{14}CH_2OH.CHNH_2.CO_2H$	L-α-Acetamido-γ-phenylbutyric acid	88·8	11·2	Pyrolysis of lithium acetate
5	D-$^{14}CH_2OH.CHNH_2.CO_2H$	L-α-Acetamido-γ-phenylbutyric acid	90·9	9·1*	Br_2/silver salt
		p-Acetamidobenzoic acid	87·1	12·9*	Br_2/silver salt
6	L-$^{14}CH_2OH.CHNH_2.CO_2H$	L-α-Acetamido-γ-phenylbutyric acid	90·7	9·3*	Br_2/silver salt
		p-Acetamidobenzoic acid	70·2	29·8*	Br_2/silver salt
8	$^{14}CH_3CO_2Na$	L-α-Acetamido-γ-phenylbutyric acid	99·0	1·0	Pyrolysis of lithium acetate
			98·4	1·6	Pyrolysis of lithium acetate

* Radioactivity calculated by difference.

isotope coefficient (RIC) and the concentration coefficient (CC) as defined by Anker (1948). The reciprocal of the concentration coefficient, called the dilution factor (Arnstein, 1951), then gives the dilution of isotope which would have been observed if exactly 1 mmol. labelled compound/100 g. body weight had been fed (see Table 4).

The results given in Tables 2–4 demonstrate that both D- and L-[β-^{14}C]-serine give rise to labelled acetyl groups, the efficiency of the conversion depending on the stereochemical configuration of the compound fed and the strain of rat used.

The distribution of isotopic tracer in the acetyl groups of the excreted acetamido acids is given in

Table 5. Pyrolysis of lithium acetate labelled only in the methyl carbon atom always gave rise to barium carbonate containing about 1·5% of the original radioactivity; the results obtained by this method have therefore been corrected to allow for this error.

DISCUSSION

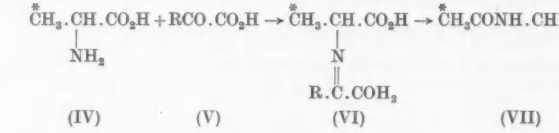
DL-Serine is deaminated both by bacterial suspensions (Gale & Stephenson, 1938) and by liver extracts (Chargaff & Sprinson, 1943*a*), the reaction taking place under either aerobic or anaerobic conditions. L-Serine appears to be converted to pyruvate by *Escherichia coli* suspensions at the same rate as the DL-compound (Chargaff & Sprinson, 1943*b*), but the D-isomer has not been investigated.

The incorporation of the β-carbon atom of serine into acetyl groups

In the present investigation the *in vivo* formation of L-α-acetamido-γ-phenylbutyric acid (APBA) and *p*-acetamidobenzoic acid (ABA) from serine was studied by feeding simultaneously [β-^{14}C]-serine, DL-α-amino-γ-phenylbutyric acid and *p*-aminobenzoic acid to adult rats and isolating the acetylated amino-acids from the urine. Pyruvic acid is known to acetylate α-amino-γ-phenylbutyric acid, but not *p*-aminobenzoic acid, whereas acetate is able to acetylate both *p*-aminobenzoic acid and α-amino-γ-phenylbutyric acid, i.e. aromatic as well as aliphatic amines (Bloch & Rittenberg, 1945).

After feeding D- or L-[β-^{14}C]-serine the isotopic tracer was found in both the excreted acetamido acids. However, quantitative differences in the conversion of the two stereoisomers to APBA and ABA were observed, especially in experiments with rats of the Institute strain, which appeared to be able to deaminate serine more readily than the P.S. strain. The specific radioactivity of the ABA was less than that of the APBA, the difference being particularly marked when D-serine was fed. In this case the specific radioactivity of the APBA was about four times that of the ABA; after feeding L-serine the radioactivity of the APBA was only 50% greater than that of the ABA.

Similar differences in acetylating capacity between the two stereoisomeric forms of alanine have been observed (Anker, 1950*a*). L-Alanine was found to give identical isotope contents in both acetamido acids, thus behaving like pyruvate (Anker, 1948), but the D-isomer gave a higher isotope content in the APBA. It was suggested by Anker that alanine is first deaminated to pyruvate, which after decarboxylation would acetylate both the aliphatic and aromatic amino-acids. The D-isomer (IV) was supposed to react first with a keto-acid (V) to give a hypothetical intermediate such as (VI) which is then converted to the acetamido acid (VII). Such an acetamido acid would be expected to transfer its acetyl group preferentially to the α-amino-γ-phenylbutyric acid, as it has been shown that acetylglycine as well as other acetamido acids, labelled in the acetyl group, give rise to a higher isotope content in the aliphatic than in the aromatic acetamido group (Bloch & Rittenberg, 1947).



(IV) (V) (VI) (VII)

The above mechanism would explain satisfactorily the difference in the ratio of the radioactivity of the aromatic and aliphatic acetamido acids after feeding D- and L-serine (see Table 4). However, degradation of the acetic acid obtained from the isolated acetyl derivatives showed that the carboxyl carbon atom of the acetic acid derived from D-[β-^{14}C]-serine contained about 10% of the radioactivity of the acetyl group. After administration of methyl-labelled acetate the carboxyl carbon atom of the acetate obtained from the excreted APBA contained only about 1% of the radioactivity. The above distribution of isotopic tracer in the acetic acid derived from D-[β-^{14}C]-serine could, therefore, hardly arise by the exclusive conversion of D-serine to an acetamido acid followed by transacetylation, as in this case all the radioactivity would be expected to be located in the methyl carbon of the acetate. Recently, it has been suggested that both stereoisomers of alanine acetylate amino-acids by the same mechanism and that differences in isotope content of the acetamido acids are due to different sites of acetylation of *p*-aminobenzoic acid and α-amino-γ-phenylbutyric acid (Anker, 1950*b*). It is pointed out that sulphanilamide (and presumably other aromatic amines, such as *p*-aminobenzoic acid) is acetylated in the liver only, but that α-amino-γ-phenylbutyric acid is acetylated also in the kidney. The kidney is known to contain high concentrations of D-amino-acid oxidase, which converts D-alanine to pyruvate, and an enzyme system which is capable of converting pyruvate to acetate. The high isotope content in APBA after feeding D-alanine is thus ascribed to the conversion of D-alanine to acetate of high isotope content and its utilization for acetylating α-amino-γ-phenylbutyric acid in the kidney. Since the acetylation of *p*-aminobenzoic acid takes place in the liver, further

dilution of the labelled acetate by the large hepatic acetic acid pool would account for the lower isotope content in ABA. Since D-serine is also oxidized to pyruvate, presumably via hydroxypyruvate (Sprinson & Chargaff, 1946) by D-amino-acid oxidase, a similar argument can be applied to the acetylation reactions of D- and L-serine. However, the almost identical distribution of isotope within the acetyl groups of both APBA and ABA after feeding D-serine (Table 5) would appear to be evidence against such an explanation, since it would be expected that the two acetylation reactions, taking place in different organs and at presumably different rates, would result in differences in the distribution of isotope in the two acetamido acids.

The present findings may be satisfactorily explained by assuming that only a part of the D-serine is converted to a C_2-fragment which acts as an acetylating agent, possibly by transacetylation, while the remainder is converted to pyruvate. L-Serine, on the other hand, is converted wholly to pyruvate, though probably not through hydroxypyruvic acid (Sprinson & Chargaff, 1946). Since pyruvate can be converted to oxaloacetate by the Wood-Werkman reaction and thence to the symmetrical dicarboxylic acids of the tricarboxylic acid cycle, any isotope originally in the methyl carbon atom of pyruvate will become partially redistributed. Hence acetate derived from methyl-labelled pyruvate will contain some isotopic label in the carboxyl group. It is probable that the tracer found in the carboxyl carbon atom of acetate after feeding [β-^{14}C]-serine arises by this mechanism.

The incorporation of the β-carbon atom of serine into glycine

The postulate that D- as well as L-serine can give rise to pyruvate is supported by the conversion of both stereoisomers, labelled in the β-position, to glycine by rats of the P.S. strain. In another experiment, using DL-[β-^{14}C]-serine and Institute strain rats the glycine, isolated as hippuric acid, had a higher isotope content (see Table 4) than in the experiments with P.S. rats. Degradation of the glycine from this experiment showed that 67% of the radioactivity was located in the α-carbon atom. Similar results have recently been quoted by Elwyn & Sprinson (1950*c*) in references to unpublished work. This finding excludes amidomalonic acid as an intermediate, as in this case only the carboxyl carbon atom of glycine would be labelled. The conversion of the β-carbon atom of serine to glycine must therefore involve deamination, randomization of the isotopic label mainly between the α- and β-carbon atoms, and reamination. Carbon atoms 1 and 2 of pyruvate are known to be precursors of glycine (probably via serine) (Anker, 1948) and the tricarboxylic acid cycle provides a mechanism for the randomization of tracer between carbon atoms 2 and 3 of pyruvate, as well as accounting for the fixation of isotope in the carboxyl carbon atom by the Wood-Werkman reaction followed by conversion to a symmetrical dicarboxylic acid, as already discussed. Furthermore, the tricarboxylic acid cycle accounts satisfactorily for the distribution of isotope in glycine after feeding labelled acetate to rats (Arnstein & Neuberger, 1949). It is probable, therefore, that the β-carbon atom of serine is converted to the α-carbon atom of glycine by deamination of serine to pyruvate, participation in the tricarboxylic acid cycle and reconversion to serine, the glycine then arising by loss of the β-carbon atom (Shemin, 1946) from the α, β-labelled serine.

After feeding β-labelled L-serine the isotope content of the excreted hippuric acid and both the acetamido acids was lower than after giving the D-isomer. This is ascribed to the dilution of the L-serine fed by endogenous L-serine. D-Serine does not appear to be synthesized by the animal to any significant extent, as the specific radioactivity of the β-carbon atom of the excreted serine was found to be the same as that of the fed D-serine.

SUMMARY

1. A synthesis of [β-^{14}C]-serine from [^{14}C]-methanol is described.

2. The conversion of serine to glycine and acetate has been studied by feeding both enantiomorphs, labelled in the β-carbon atom with ^{14}C, to adult rats and isolating hippuric acid, L-α-acetamido-γ-phenylbutyric acid and *p*-acetamidobenzoic acid from the urine.

3. It is concluded that both enantiomorphs of serine are precursors of acetyl groups *in vivo*.

4. The mechanism of this conversion is discussed.

I wish to thank Dr A. Neuberger, F.R.S., for many helpful discussions during the course of this work, Dr T. S. Work and Dr P. N. Campbell for advice concerning the chromatographic isolation of serine, and Mr A. Spiers for valuable technical assistance.

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The Action of a Hydrolytic Enzyme System from *Helianthus tuberosus* L. on Carbohydrates Present in the Tubers

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Green (1887) described an 'inulin-ferment' (later named inulase: Green, 1893) which was present in glycerol extracts made from sprouting tubers of the Jerusalem artichoke and which liberated from inulin reducing sugar and uncharacterized intermediate products. Wolff & Geslin (1918) made from chicory roots extracts in which reducing sugar was slowly liberated, and Colin (1919) reported a similar observation with extracts of artichoke tubers; in both cases the liberation of reducing sugar was correlated with a change of optical rotation in the negative direction. Pringsheim & Kohn (1924) attempted to prepare enzymes from dahlia tubers, but considered their efforts unsuccessful. Thaysen, Bakes & Green (1929) were led to suspect the presence of an inulase in artichoke-tuber extract, but could not confirm this experimentally. These various findings have led to the general conclusion that higher plants are not good sources of enzymes attacking inulin (Pringsheim & Leibowitz, 1929; cf. Lemoigne, 1942).

However, Vadas (1934) suggested the use of a short incubation of mashed tubers as a preliminary to ethanolic fermentation of the carbohydrates, and Asai (1938) noted that as much as 50 % of the carbohydrate in the tubers was converted to reducing sugar when the crushed material was incubated for 20 hr. at 37° and pH 3·9.

Physiological studies of the artichoke plant (cf. Colin, 1919; Belval, 1946) indicate that enzyme systems exist concerned with the transformation of the tuber carbohydrates. The latter consist of a mixture of related oligo- and poly-saccharides as well as inulin itself. In a previous paper (Bacon & Edelman, 1951) we have described the application of paper partition chromatography to these substances.

During that investigation we re-examined the possibility of preparing from the tubers extracts with enzymic activity towards inulin and related substances. The results presented here demonstrate the existence in tuber extracts of a hydrolytic enzyme system, which liberates fructose from inulin and related substances, but has relatively little action upon sucrose.

Part of this work was communicated to the Biochemical Society at Leeds on 23 September 1949 (Edelman & Bacon, 1949).

MATERIALS AND METHODS

Except where otherwise stated, the methods of analysis used were those described by Bacon & Edelman (1951), as also were the batches of artichoke tubers. Reducing substances (RS) are expressed in terms of hexose and by 'total RS' is meant RS after hydrolysis with 0·5 % (w/v) oxalic acid for 30 min. at 100°. The sample of commercial inulin used was obtained from Messrs Thomas Kerfoot and Co., Vale of Bardsley, Ashton-under-Lyne, who kindly informed us that it was prepared from the root of the dandelion, *Taraxacum officinale*. All samples of inulin were purified by filtering the hot aqueous solution, freezing the filtrate, dehydrating the resulting precipitate with ethanol and acetone, washing it with light petroleum and drying it in a vacuum desiccator.

The lead acetate and sodium oxalate solutions used throughout were: 30 % (w/v) $Pb(C_2H_3O_2)_2.3H_2O$ and 3 % (w/v) $Na_2C_2O_4$. All dialysis was carried out in Visking synthetic cellulose casing (John Crampton and Co. Ltd., Wythenshawe, Manchester).

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RESULTS

Characterization of enzymic activity

When tuber extracts made as described by Bacon & Edelman (1951) were incubated at 37–40°, their optical rotation was found to change in the negative direction, and the free RS to increase. There was no change in the RS liberated by mild acid hydrolysis (Table 1). The quantitative relations between change in RS and change in optical rotation were consistent with the liberation of fructose from laevorotatory oligo- or poly-saccharides.

Table 1. *Changes in reducing substances (RS) and optical rotation on incubation of tuber extract*

(Fresh extract, 17 November 1948, was incubated at 40°, and samples taken for deproteinization and measurement of free and total RS, and rotation (2 dm. tube).)

Time of incubation (hr.)	Optical rotation	RS (mg./ml.) Free	RS (mg./ml.) Total
0	−2·73°	3·88	—
4·5	−3·20°	8·17	133
22	−4·75°	20·5	136

The same changes were observed when extracts were incubated in the presence of toluene or $CHCl_3$, and microscopical examination of incubated extracts, either by dark-ground illumination or after the use of Gram stain, showed that no significant growth of micro-organisms had occurred. The effect observed must therefore be attributed to constituents of the tubers; it was abolished when the extracts were boiled.

The activity was not affected by the presence of cyanide in concentrations up to 0·005 M.

In order to distinguish between phosphorolytic and hydrolytic breakdown of carbohydrate, incubations were carried out with the addition of inorganic phosphate. A disappearance of inorganic phosphate under these conditions would suggest phosphorolysis; an increase in the rate of RS liberation without a change in inorganic phosphate might be interpreted as being due to the simultaneous action of a phosphorylase and a phosphatase. Phosphatase activity was present in the extracts, as was shown by the following experiment: 0·5 ml. of extract, diluted to 2·5 ml. with water, was incubated with 5 ml. 0·01 M-disodium phenyl phosphate, and either (*a*) 5 ml. 0·4 M-sodium acetate buffer (pH 5·0) plus 0·2 ml. water, (*b*) 5·2 ml. water, giving a pH value of 6·5, or (*c*) 5·0 ml. water plus 0·2 ml. M-NaF, giving pH 6·5. The phenol liberated during 1 hr. incubation at 40° was estimated as described by King (1946), amounting to (*a*) 0·65 mg., (*b*) 0·056 mg., (*c*) 0·003 mg. Experiments to detect phosphorolytic breakdown of polysaccharide were therefore carried out in the presence of 0·02 M-NaF, in order to reduce any effects of phosphatase to a minimum.

Several portions of extract were incubated at 26° for 40 hr. and samples taken at intervals for estimation of RS and inorganic phosphate (by the method of Fiske & Subbarow, 1925). No differences from controls without added fluoride or inorganic phosphate were observed at pH 6·4 with 0·01 M added phosphate nor at pH 7·5 with 0·02 M-phosphate (see Table 2); the extract already contained about 0·005 M-inorganic phosphate. The increase in RS and inorganic phosphate during intermediate periods showed no differences from the overall change given in Table 2. At pH 6·9 with 0·17 M added phosphate there was a decrease of 18% in RS liberation both with and without fluoride, but no corresponding decrease in inorganic phosphate; it is possible that the enzyme system was inhibited by the high concentration of phosphate.

The above results suggest that the activity of the tuber extracts is due to a hydrolytic, rather than a phosphorolytic, system (see also experiments with inulin, described below).

Measurement of activity

Since it was not known upon what substances the enzyme or enzymes concerned were acting, the possibility existing that inulin itself was not attacked, it was thought advisable to use as a substrate for measuring enzyme activity the whole mixture of soluble carbohydrates present in the tubers, and to define a unit of activity as the liberation of 1 mg. of RS (calculated as hexose) from such a mixture under conditions defined below.

Preparation of standard substrate. Washed tubers (350 g.) (bought and extracted 8 February 1949) were extracted in the usual way in the Waring blender. The extract obtained was used for the extraction of a further 350 g. Four such double extracts were made, yielding 2·17 l. This liquid was heated in a boiling-water bath for 10 min., 0·25 vol. M-sodium acetate buffer (pH 5·0) was added and the resultant liquid filtered after 10 min. Analyses gave values of 2 mg./ml. RS in the unhydrolysed filtrate, 101 mg./ml. RS after mild acid hydrolysis, and 80 mg./ml. total ketose. The liquid was clear and light yellow; when saturated with toluene it was stored in the refrigerator for several months without

Table 2. *Changes in reducing substances (RS) and inorganic phosphate during incubation of tuber extracts*

(An extract, made 14 November 1949, was incubated with and without 0·02 M-sodium potassium phosphate buffer (pH 7·5), with and without 0·02 M-NaF, the pH of all mixtures being adjusted to 7·5. After 50 hr. at 26° the reaction was stopped by heating the samples in a boiling-water bath.)

		mg./ml. incubation mixture			
		No added phosphate		0·02 M added phosphate	
		With NaF	No NaF	With NaF	No NaF
RS:	0 hr.	1·6	1·8	1·6	1·7
	50 hr.	10·1	10·7	10·6	9·8
	Change	8·5	8·9	9·0	8·1
P of inorganic phosphate:	0 hr.	0·11	0·11	0·67	0·68
	50 hr.	0·18	0·19	0·72	0·72
	Change	0·07	0·08	0·05	0·04

detectable change in the appearance or analysis. This buffered extract is subsequently referred to as 'standard substrate'. The optical rotation and chromatographic analysis of the various 'spots' of this preparation are given by Bacon & Edelman (1951; Tables 1 and 4).

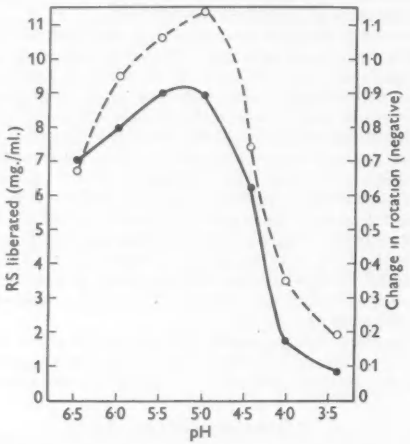

Fig. 1. Effect of pH on the liberation of reducing substances (RS) and the changes in optical rotation in tuber extracts. Samples of extract (10 December 1948) were adjusted to the required pH value with N-HCl and changes in RS and optical rotation (2 dm.) were measured on incubation for 4 hr. at 37°. ●—●, RS; ○- - -○, optical rotation.

Effect of pH. The effect of pH on the change in RS and optical rotation during incubation is shown in Fig. 1. The pH of the extract was altered by adding N-HCl. During incubation there were small changes in pH, the greatest being from 5·98 to 6·14, but in other cases less than 0·1 unit. The maximum activity occurred at about pH 5, with only slight decrease at higher, and a more marked decrease at lower, pH values.

Progress curve of the reaction. During the early stages of the reaction liberation of RS was proportional to the time of incubation (Table 3).

Table 3. *Initial stages of liberation of reducing substances (RS) from standard substrate (tuber extract)*

(Enzyme preparation (made 29 March 1950, see Fig. 4) incubated as for activity measurement and samples withdrawn at intervals for estimation of total RS.)

	In 6 ml. incubated mixture	
Time of incubation (hr.)	RS (mg.)	Liberation of RS (mg.)
0	2·43	0
1	5·67	3·24
2	9·35	6·92
3	11·9	9·47

Effect of substrate concentration. An enzyme preparation free from carbohydrates was made from a tuber extract (3 February 1949) by $(NH_4)_2SO_4$ precipitation at 60% saturation. Another portion of the extract was heated for 15 min. in a boiling-water bath, cooled, and diluted with 0·25 vol. M-sodium acetate buffer (pH 5·0); it contained 2·3 mg./ml. of free RS, 93·8 mg./ml. total RS, and 75 mg./ml. ketose. Dilutions with 0·2 M-acetate buffer were used as substrates for the action of the enzyme preparation. The results (Fig. 2) indicate that rates of reaction approaching the limiting velocity are reached with concentrations of about 75 mg. total RS/ml.

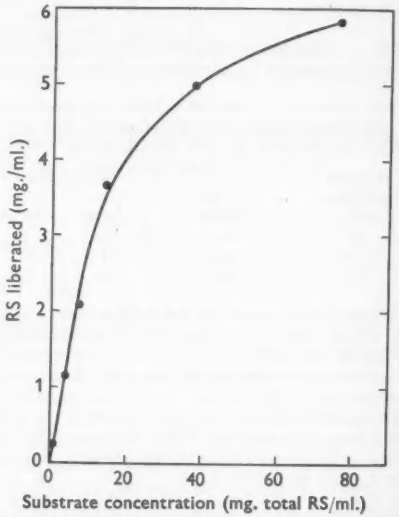

Fig. 2. Effect of carbohydrate concentration on the rate of liberation of reducing substances (RS) from tuber extracts. A quarter volume of enzyme preparation was added to boiled extract diluted as indicated (both preparations described in Text) and incubated for 3 hr. at 40°. The liberation of RS, expressed as mg./ml. is corrected for the RS content of the enzyme preparation, 0·21 mg./ml.

Effect of enzyme concentration. A preparation made by precipitation with $(NH_4)_2SO_4$ was added in varying amounts to standard substrate and incubated for 3 hr. at 37°. The amounts of RS liberated are shown in Table 4.

Unit of activity. Consequent upon the above experiments 1 unit of activity was defined as that which liberates 1 mg. RS (calculated as hexose on the basis of a fructose standard) from standard substrate diluted from 5 to 6 vol. (thus containing 84 mg. total RS/ml.) at pH 5·0 in 0·17 M-sodium acetate buffer in 180 min. at 40°.

Estimation of enzyme activity. For the estimation of enzyme activity 0·5 ml. of the enzyme solution was mixed with 2·5 ml. standard substrate, and two 1·0 ml. samples were transferred to test tubes. (In the case of solutions with low activity, i.e. less than 10 units/ml., 1·0 ml. was mixed with 2·0 ml., the remainder of the procedure being the same.) One of the tubes was incubated at 40° for 2 min. and then heated in a boiling-water bath for 3 min. to inactivate the enzymes. The other was incubated for between 32 and 182 min. according to the approximate enzyme activity and

then treated similarly. After cooling 0·1 ml. lead acetate solution was added to each tube and they were allowed to stand for 10 min. The solutions were filtered through a Whatman no. 1 paper (diameter 5·5 cm.) and the filter paper containing the precipitate was washed at least three times with several ml. of distilled water. To the combined filtrate and washings was added 0·5 ml. sodium oxalate solution, the precipitate of lead oxalate being filtered off and washed as before. The final filtrate was made up to known volume (50 or 100 ml.) for estimation of RS.

Table 4. *Relation between enzyme concentration and rate of liberation of RS*

(Different amounts of an enzyme preparation, prepared 21 February 1949 by a single precipitation between 25 and 55% saturation $(NH_4)_2SO_4$ (activity: 33 units/ml.) were incubated with 5 ml. of standard substrate for 3 hr. at 37°. Water added to make vol. 6 ml.)

Vol. enzyme preparation in 6 ml. incubation mixture (ml.)	RS liberated (mg./ml.)	RS liberated/ml. enzyme preparation (mg./ml.)
0·0	−0·40	—
0·2	0·95	41
0·4	1·79	33
0·6	2·81	32
0·8	3·71	31
1·0	5·17	34
2·0*	9·14	29

* 4 ml. substrate used.

In preliminary experiments on activity estimation, it was assumed that addition of lead acetate to the solutions used in these estimations was sufficient to inactivate the enzyme. It was found, however (Bacon & Edelman, 1951), that the optical rotation of tuber extracts deproteinized by adding 0·1 vol. lead acetate solution changed slowly in the negative direction, no such change occurring in extracts boiled before deproteinization. Unboiled deproteinized extracts were perfectly clear and almost colourless, but gave a slight haziness with 5% (w/v) aqueous trichloroacetic acid. The failure of lead to remove the enzyme system is illustrated by the following experiment.

To four samples *A*, *B*, *C* and *D* of an enzyme preparation were added 0·1 vol. of 30, 22·5, 15 and 7·5% lead acetate solution respectively. The solutions were centrifuged, and the enzyme activities of the supernatant fluids were estimated: these were 18·0 (control), 7·8 (*A*), 9·6 (*B*), 10·8 (*C*) and 11·4 (*D*) units/ml. After this observation all solutions in which enzyme inactivation was required were immersed in boiling water for at least 3 min.

Example of estimation of activity. Standard substrate (2·5 ml.) incubated with 0·5 ml. enzyme concentrate. RS in 1·0 ml. samples incubated for 2 and 62 min. were 3·50 and 5·65 mg. respectively. Increase during 60 min.: 2·15 mg./ml. The activity is obtained by multiplying this value by the time factor 3, and by the enzyme dilution factor 6, giving 39 units/ml. enzyme solution.

Preparation of enzyme concentrates

In the first stages of attempts to prepare enzyme concentrates the estimations of activity were not exactly comparable, because the original extract contained large amounts of carbohydrate.

The concentration of substrate was thus higher than in the estimations at later stages when the contaminating carbohydrate had been largely or completely eliminated. As the activity of the initial extracts was low, the concentration of standard substrate was decreased by the necessity of adding a larger proportion of enzyme solution in order to give a measurable increase of RS; this may have counterbalanced the first effect to some extent, but in any case the alteration in substrate concentration was not large enough to introduce any serious error.

The concentration of activity by fractional precipitation with $(NH_4)_2SO_4$ is illustrated by the following preparation (7 April 1949). Five batches of 400 g. scrubbed tubers were disintegrated in the Waring blender. Water was added to the first batch only, sufficient liquid from this being used to extract the next batch and so on. During each extraction 5 or 10 ml. 0·2 M-KCN were added to inhibit phenol oxidases. The mash obtained after each blending was squeezed through gauze, and the extracts combined to give a total of 1·28 l. A sample was removed for estimation of enzyme activity (5·5 units/ml.) (stage 1).

To 1·27 l. (total units 6970) were added 183 g. powdered $(NH_4)_2SO_4$ (A.R.) with stirring (0·25 saturation), N-NaOH being added dropwise to keep the solution approximately neutral (green to bromothymol blue). The liquid was filtered on four large Büchner funnels using thick pads of 'Hyflo Supercel' (Johns Manville Co. Ltd., Artillery Row, London, S.W. 1), on Whatman no. 1 filter paper; filter aid was also added to the solution. Yield: 1·29 l. clear filtrate having activity, 4·6 units/ml. (estimated on dialysed sample, corrected for volume increase during dialysis) (stage 2).

To 1·28 l. (total units 5890) were added 246 g. $(NH_4)_2SO_4$ (0·55 saturation) and the filtration was carried out on a single large Büchner funnel using filter aid as above. The filter cake was sucked as dry as possible without passage of air, turned out on to a sheet of filter paper sprinkled with Hyflo Supercel, and pressed between filter papers until it crumbled readily. It was ground in a mortar with 30 ml. water and centrifuged. The precipitate was washed three times by centrifugation with about 20 ml. water, and the initial supernatant fluid and the washings were combined to give 90 ml. This liquid was dialysed for 1·5 hr. against running tap water at 8°. The volume after dialysis was 144 ml. The slight precipitate which had formed was removed by centrifugation and rejected. Activity of supernatant, 26·3 units/ml. (stage 3).

To 91 ml. (total units 2400) were added 30·3 ml. neutralized saturated $(NH_4)_2SO_4$ solution, and to 116 ml. supernatant after centrifugation were added a further 77·5 ml. saturated $(NH_4)_2SO_4$ solution. On centrifuging a loosely packed precipitate formed, to which were added 2 ml. of water to give 13 ml. of enzyme concentrate. Activity, 114 units/ml. (corrected after dialysis). Total units 1480 (stage 4).

The final concentrate was a viscous suspension in approximately half-saturated $(NH_4)_2SO_4$ solution, and was stored in the refrigerator for several weeks with only slight loss in activity. Small portions were dialysed overnight before use, the dialysed solution being an opaque mobile liquid. No further attempt was made to purify the enzyme system.

Unless an enzyme preparation with high activity was required experiments were carried out using preparations

corresponding to stage 3. Activities of between 30 and 40 units/ml. could be obtained at this stage by centrifuging the solution at fairly high speed (about 3000 *g*) instead of filtering it, and taking up the precipitate in a minimum of water.

Nature and specificity of enzymic action

The action of enzyme concentrates was tested on a number of substances, including, in addition to the tuber carbohydrates, sucrose, inulin, and two other fructose-containing polysaccharides.

Action on the soluble carbohydrates of the tuber. Paper partition chromatograms of samples taken during the incubation of tuber extracts showed that free fructose and traces of glucose were formed. It appeared, therefore, that the appearance of free RS was probably caused by the hydrolysis of non-reducing higher saccharide to reducing monosaccharide, and it became of interest to study the final products of this action.

When the enzyme concentrate described above was incubated at 40° with standard substrate so diluted as to give a concentration of 10 mg. combined RS/ml. detectable appearance of RS ceased before the theoretical value for total acid hydrolysis (Fig. 3). The amount not appearing in this experiment was 33·5% of the combined RS. After 50 hr. standard substrate was added to the solution to bring the concentration of combined RS approximately to the initial value. Free RS was estimated immediately, and after incubation for a further 2 hr. That the enzyme was still active was shown by the appearance of a further 1·43 mg. RS/ml. during this period, although the activity was now less than at the beginning of incubation, when 4·2 mg. RS/ml. had been liberated in an equal time. Addition of 16 mg. fructose/ml. at 50 hr. had no significant effect on the RS already present, the values being 24·2 mg. RS/ml. immediately after the addition, and 24·5 mg. RS/ml. 2 hr. later. It seemed probable, therefore, that the cessation of hydrolysis was due to part of the combined RS not being attacked by the enzyme, and not to an equilibrium with the fructose liberated.

The nature of this unattacked material was investigated chromatographically. Qualitative chromatography indicated the disappearance of all spots with R_F values lower than that of sucrose. A large amount of fructose, but little or no glucose, had appeared and there was an apparent increase of 'spot 1'. These findings were borne out by quantitative analysis of chromatograms from a similar experiment (Table 5). It had been shown (Bacon & Edelman, 1950*b*, 1951) that the spot 1 initially present in tuber extracts is mainly, if not all, sucrose. That the material causing the

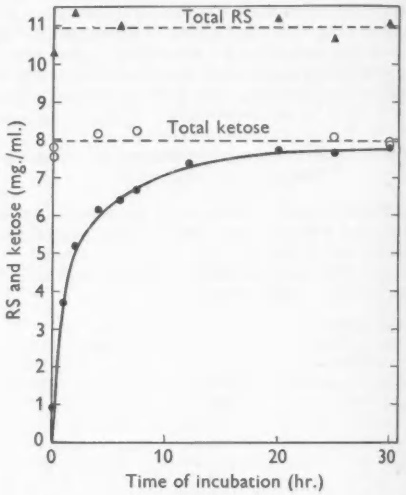

Fig. 3. Course of liberation of reducing substances (RS) from artichoke extract. Standard substrate, diluted with 0·2 M-sodium acetate buffer (pH 5·0) to give 12 mg. combined RS/ml. (12 ml.), was incubated at 37° with 2·5 ml. of enzyme preparation (7 April 1949, described in text). RS, before (●) and after (▲) hydrolysis, and ketose (○), were estimated on 1·0 ml. samples.

increase in this spot during incubation with the enzyme system may also be sucrose was indicated by the following:

(*a*) The theoretical reducing sugar values calculated from the fructose content for this spot (assuming it to be sucrose) were 458 and 480 μg./20 μl. in the 600 and 1980 min. samples respectively. The values given after extraction from the paper and hydrolysis with invertase were 440 and 438 μg./20 μl. (4 ml. samples of the extracts were incubated for 90 min. at 40° with 1 ml. of a 1–25 dilution of British Drug Houses Ltd. 'Invertase Concentrate', which had been

Table 5. *Effect of hydrolytic enzyme preparation on the components of tuber extract*

(0·5 ml. standard substrate was incubated at 40° with 0·5 ml. of an enzyme preparation, prepared 16 January 1950 by a single precipitation between 25 and 52% saturation $(NH_4)_2SO_4$, activity 36 units/ml., dialysed before use. The ketose contents of the individual components were followed as previously described (Bacon & Edelman, 1951).)

	Time of incubation (min.)						
Spot	0	60	200	360	600	1440	1980
	μg. ketose/20 μl. incubation mixture						
F	—	97	240	384	500	563	590
1	91	101	140	177	229	254	240
2	87	87	92	77	51		
3	97	95	74	47			
4	87	70	40	24	92	29	27
5	84	66	40	24			
6 to *n*	392	307	220	124			
Total	838	823	846	857	872	846	857

dialysed overnight against running tap water; no attempt was made to remove the enzyme before estimation of RS. A paper 'blank' was treated similarly; it amounted to about 25% of the RS measured.) These values, assuming complete breakdown by invertase, give ratios of fructose : glucose of 1:1·02 and 1:1·13.

(*b*) Only traces of free glucose appeared on the chromatogram even at 1980 min. incubation, and all the glucose in the extract could be accounted for by the material in spot 1, if that was assumed to be sucrose (as in the following calculations). Total fructose in the 1980 min. sample was 857 μg./20 μl., therefore total RS should be 857 + 240 = 1097 μg./20 μl., making the glucose as percentage of combined RS = (240/1097) × 100 = 21·8. Similarly, the glucose in the 1440 min. samples would constitute 22·7% of the combined RS. The determined value for this extract was 21%.

(*c*) The enzyme preparations had very little action on sucrose (see below).

Action on sucrose. Enzyme concentrates were only very weakly active against sucrose under the conditions of temperature and pH normally used. Thus an enzyme preparation acting on standard substrate with activity 99 units/ml., had an activity of only 4·7 units/ml. against a sucrose solution with the same concentration (w/v) of combined RS. As the tuber extract material probably has a mean molecular weight of the order of 1000 at least, the molar concentration of sucrose was considerably greater than that of the standard substrate.

Action on fractions of standard substrate. To investigate the action of enzyme preparations on the components of the standard substrate with higher R_F, five fractions (*A* to *E*) were separated from standard substrate by paper partition chromatography in phenol followed by Soxhlet extraction from the paper. (For further details of these fractions see Bacon & Edelman, 1951.) Fraction *B* consisted of a mixture of spots 2 and 3 and contained 38·7% glucose, 61·3% fructose. Fraction *D* had R_F close to zero and probably contained more than two components. It contained 10·3% glucose, 98·7% fructose. Fraction *B*, on incubation with an enzyme concentrate of activity about 100 units/ml., showed progressive disappearance of spots 2 and 3 with time and a corresponding appearance and increase of spots in the same positions as spot 1 and fructose. At 180 and 360 min. incubation there was little or no carbohydrate material visible with R_F less than that of spot 1. At 1080 min. spot 1 itself had largely disappeared. The fructose spot increased throughout the incubation. Glucose was not shown by the spraying reagent used (phloroglucinol). Fraction *D* showed spots 1–3 and a higher streak on incubation with the enzyme, and an even greater quantity of fructose than fraction *B*. The weakness of spots 1–3 could be ascribed to the small amount of combined glucose in this fraction, as these three spots contain a relatively high glucose : fructose ratio, spot 1 probably being sucrose. These intermediate spots eventually disappeared except for spot 1, which was present even at 1080 min.

The R_F values of the substances detected in these experiments cannot be taken as proof of their identity with the spots of the original tuber extract, and the possibility is not excluded that some at least of the material in these positions may have been composed entirely of fructose residues.

Action on inulin. The rate of liberation of reducing sugar from standard substrate by the action of an enzyme concentrate was greater than that from an equivalent concentration of inulin (w/v) under the same conditions of pH and temperature. The molecular weight of inulin is, however, probably considerably greater than that of the mean molecular weight of the carbohydrates in standard substrate, so that its molar concentration would be correspondingly lower. However, no constant relationship was found between activity against standard substrate and activity against inulin. This variability, which was not fully investigated, may have been due to variation in the degree of aggregation of inulin in the incubated mixture; on prolonged incubation inulin was often seen to precipitate.

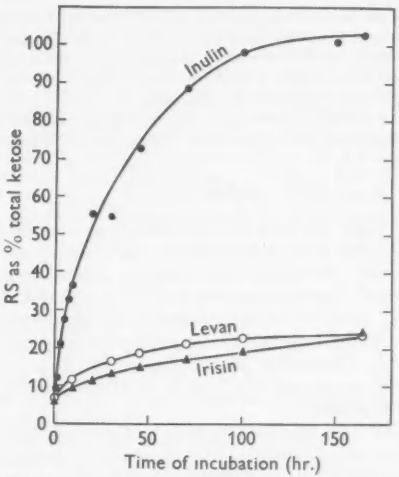

Fig. 4. Liberation of reducing substances (RS) from dandelion inulin, grass levan and irisin 'B'. Approximately 4% (w/v) solutions of the polysaccharides in 0·2 M-sodium acetate buffer (pH 5·0) were incubated at 40° with equal volume (7 ml.) of an enzyme preparation, made 29 March 1950 by a single precipitation between 20 and 50% saturation $(NH_4)_2SO_4$ (activity: 58 units/ml.). The solutions lost water by evaporation during the long incubation, so the RS is plotted as percentage of total ketose.

The action on dandelion inulin at pH 5 (see also Fig. 4), when investigated by qualitative chromatography, was found to produce a large fructose spot, a trace of glucose, a spot identical in R_F (butanol-acetic acid) with sucrose, and a much weaker spot apparently in the same position as spot 2 of artichoke extracts. The spot in the sucrose position became progressively stronger with increasing time of incubation. Its colour with the benzidine-trichloroacetic acid reagent was at first the typical yellow given by fructose, but became darker brown and thus more suggestive of the presence of combined glucose. These observations were corroborated by two-dimensional chromatography using an invertase spray between developments (see Bacon & Edelman, 1951). At first the substances in the positions of spots 1 and 2 showed no glucose, but after longer incubation significant amounts of glucose were detected in the spot 1 position, suggesting that sucrose is produced from inulin and may be an integral part of its molecule. Control incubations of buffered enzyme, alone and with a mixture of fructose (10% w/v) and glucose

(0·3% w/v), gave no formation of a spot in the sucrose position.

Inorganic phosphate, at a concentration of 0·02 M (as KH_2PO_4), had no effect upon the liberation of RS from inulin in 0·17 M-sodium acetate buffer (pH 5·0) during 24 hr. at 40°. There was no change in inorganic phosphate during the first 10 hr. of incubation.

No inorganic phosphate was detected at any stage during the control incubation, in contrast to the control experiment with fresh extract in which inorganic phosphate was present initially, and increased significantly during incubation (Table 2).

Action on irisin and grass levan. The liberation of RS by the action of enzyme concentrate on irisin 'B' (kindly supplied by Dr D. J. Bell) and a levan prepared from a dried sample of Italian rye-grass is shown in Fig. 4. The breakdown of inulin under the same conditions is also shown. Control incubations of the three polysaccharides in the absence of the enzyme showed no significant appearance of RS during 24 hr.

DISCUSSION

Although the action of inulases from moulds has been studied fairly extensively, there have been few reports of investigations of higher plants since the original observations of Green (1887). Pringsheim & Kohn (1924) referred to attempts to extract inulase from inulin-bearing plants but gave no experimental details. Neither Wolff & Geslin (1918) nor Asai (1938) examined the action of their extracts on inulin alone.

The preparations described here were fully as active on a volume basis as the most active preparations from *Aspergillus niger* cited by Pringsheim & Kohn (1924), and the activity of fresh extracts of tubers, made in the Waring blender, was measurable over relatively short periods of incubation (2–3 hr.). There would thus seem to be no practical objection to using the tissues of higher plants as sources of inulase; there is also the advantage of working with tissues which normally contain and presumably metabolize the polysaccharide.

The purest yeast-invertase preparations so far studied (Adams, Richtmyer & Hudson, 1943) showed some hydrolytic activity towards inulin, although it is not certain (cf. Pigman, 1944) whether this is to be ascribed to an unspecific action of invertase itself, or to a contaminating enzyme. The mould preparations studied by Pringsheim & Kohn (1924) had appreciable invertase activity which was diminished to a lesser extent than the inulase activity by adsorption on calcium phosphate. The preparations described here were never altogether free from action upon sucrose, but the rate of liberation of fructose and glucose from this substance was never more than 20% of the rate of fructose production from inulin at similar concentrations (w/w). Pringsheim & Kohn (1924) found three times as much invertase as inulase activity in their preparation. It may be significant that no substances other than sucrose, glucose, and fructose were seen on chromatograms when the artichoke preparations acted upon sucrose, in contrast with observations on yeast invertase (Blanchard & Albon, 1950; Bacon & Edelman, 1950*a*).

The chromatographic studies of the action of tuber preparations show that fructose is the first product of their action on inulin; the appearance of fructose-containing substances in the di- and tri-saccharide positions fairly early in the hydrolysis may be due to a transfructosidation reaction (Edelman & Bacon, 1950) between the fructose formed and the inulin not yet broken down. It would thus seem that the enzyme preparation has the properties of a fructofuranosidase and that it is most active towards the 2-1′-linked terminal residue of inulin and related compounds. The crude preparations employed in the present investigation possessed a limited activity towards grass levan and irisin 'B', two other polysaccharides consisting chiefly of fructose. The irisin molecule is highly branched (Bell & Palmer, 1949), having both 2-1′ and 2-6′ linkages, though its exact structure cannot be decided upon. The slow rate at which it is attacked by the artichoke preparation might be taken as suggesting that its 'backbone' is linked in the inulin manner, while the side groups have the levan linkage, the action of the enzyme thus being limited by the speed with which the side groups could be removed to expose the more vulnerable 2-1′ linkage.

A preliminary study by Miss M. Holden, at Rothamsted Experimental Station, of the action of certain fungal enzyme preparations (cf. Holden, 1950) on inulin, grass levan and irisin showed similar results, in that the degree of hydrolysis of irisin was in each case similar to that of the levan (Holden, 1949). The preparations with most inulase activity were most active against the other two polysaccharides, but in every case the rate of liberation of reducing sugar from inulin was considerably greater. A bacterial levan gave results similar to those with the grass levan.

The slow but significant hydrolysis of sucrose by our preparations might be ascribed to the action of a not wholly specific fructofuranosidase, the converse of the inulase activity of invertase preparations. However, clarification of this and other problems concerning specificity and the more important problem of the relation between hydrolysis and transfructosidation in artichoke-tuber preparations must await the results of a systematic attempt to purify the enzyme or enzymes concerned.

From the metabolic point of view the present results do not represent any advance on the original discovery of Green (1887), which conformed to the general observation that reserve carbohydrates may often be hydrolysed by extracts of the tissues which

contain them. It is notable, though, that the artichoke preparations described here had very little hydrolytic action upon sucrose, which was present in concentrations up to 1·5% (w/w fresh tuber) in all the tubers examined.

SUMMARY

1. Aqueous extracts of tubers of the Jerusalem artichoke (*Helianthus tuberosus*) show, on incubation, an increase in reducing substances and a decrease in optical rotation.

2. The enzyme or enzymes responsible have been freed from the tuber carbohydrates and concentrated twenty-fold by fractional precipitation with ammonium sulphate.

3. By the use of paper partition chromatography such preparations have been shown to liberate fructose from various substrates, including sucrose, inulin, a levan, and irisin 'B'; glucose was detected among the hydrolysis products of inulin.

4. The liberation of reducing sugars from the tuber carbohydrates and from inulin virtually ceased before complete hydrolysis had taken place. The residual combined sugar was in the form of a disaccharide, probably sucrose.

5. From these observations the main hydrolytic activity of tuber preparations has been characterized as that of a fructofuranosidase, with a moderate degree of specificity for such residues linked to position 1 of another fructose residue.

This work was done with the technical assistance of Mr R. Loxley. We wish to thank Prof. H. A. Krebs, F.R.S., for helpful discussion. We are grateful to Miss M. Holden for allowing us to see the results of unpublished experiments; to Mr G. S. Horner, of the Department of Education of this University, for a translation from the original Japanese of the paper by Toshinobu Asai; to Dr T. H. M. Kerfoot for information about the inulin that we used; and to Dr D. J. Bell for a sample of irisin 'B'. One of us (J. E.) is indebted to the Agricultural Research Council for grants during the course of the research.

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The Metabolism of Pyruvate by *Lactobacillus plantarum*

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When pantothenate is added to suspensions of *Lactobacillus plantarum* deficient in that factor the yield of acetylcholine is much increased (Stephenson & Rowatt, 1947). Hills (1943) showed that the rate of oxidation of pyruvate by pantothenate-deficient suspensions of *Proteus morganii* is increased by the addition of pantothenate. It was thought on the basis of these observations that pantothenate might be required for the metabolism of pyruvate in *Lb. plantarum*, and an investigation of the latter was planned. Pyruvate was found to be broken down to acetoin and carbon dioxide without the participation of pantothenate. This finding and various other aspects of pyruvate metabolism in this organism are described in the present paper.

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METHODS

Growth of organisms. (1) Lb. plantarum. The organism used in the main part of this work was that isolated by Stephenson & Rowatt (1947). It was maintained on medium 1 of that paper at 25°. Cells for use as washed suspensions were grown at 25° in basal medium 5 of the same paper (basal medium 5: acid digest of casein supplemented with tryptophan, cysteine, glucose, sodium acetate, salts and growth factors including pantothenate and aneurin). The following alterations in composition were made: $MgSO_4.7H_2O$ (10 mg./100 ml.) and $MnSO_4.5H_2O$ (0·025 mg./100 ml.) were autoclaved separately and added to the medium after sterilization; calcium pantothenate was sterilized by filtration through a Seitz filter and added to the sterile medium at a concentration of 10 μg./100 ml.; yeast extract was sterilized by filtration through a Seitz filter and 3 ml. added to each 100 ml. of sterile medium. The yeast extract was prepared by heating 500 g. baker's yeast in 500 ml. water for 15 min. in a boiling-water bath and filtering. When added to the medium at this concentration it increased by approximately 7 times the crop of cells obtained after 15 hr. incubation. The medium with these modifications will be referred to as medium *P* and was used for growth in all cases except where otherwise stated.

A medium deficient in pantothenate was obtained by omitting the pantothenate supplement and using yeast extract which had been autoclaved for 15 min. at 15 lb. pressure with 0·1 vol. 10 N-HCl. Growth with the acid-treated extract in the absence of added pantothenate was poor (0·06 mg. cells/ml. instead of 0·8 mg./ml. after 48 hr. incubation), but when pantothenate was added the same stimulation was found with acid-treated as with the untreated extract. As the crop of cells obtained from the pantothenate-deficient medium was small, one or more litres of medium were inoculated for each experiment. Each litre flask was inoculated with a 24 hr. culture in 100 ml. medium *P* containing 0·01 μg. calcium pantothenate/ml.

Aneurin-deficient cells were grown in medium *P* omitting both the aneurin supplement and the yeast extract.

Cultures were grown in 100 ml. lots of medium *P* in flat-bottomed flasks filled to the neck, as a smaller crop of less active cells was obtained under aerobic conditions and the yield was not increased by strict anaerobiosis. When larger quantities of cells were required, for the experiments at pH 6 for example, 500 ml. or more of medium were inoculated with 0·1 vol. of a 24 hr. culture in the same medium. Cells for washed suspensions were harvested after 15–18 hr. growth, because, although growth continued until the 45th hour, the younger cells were more active in washed suspensions.

Suspensions of washed cells in saline lost the capacity to form CO_2 from pyruvate on storage even at 4°, and they were generally used as soon as possible and never more than 5 hr. after removal from the medium.

(2) Aerobacter aerogenes *and* Staphylococcus aureus. *A. aerogenes* strain 240 (National Collection of Type Cultures no. 418) and *Staph. aureus*, strain E (McIlwain & Hughes, 1948) were grown for 15 hr. at 25–30° in 100 ml. medium containing: 10 ml. Stephenson's inorganic medium (Stephenson, 1939), 0·3% Pronutrin (Herts Pharmaceuticals Ltd.), 0·1 M-glucose and 3 ml. yeast extract (prepared as described above). The pH of the medium was adjusted to 6·5 before incubation.

Preparation of washed suspensions. After incubation, cells were centrifuged from the medium, washed once with and resuspended in 0·9% NaCl to give a suspension containing 0·5–3·0 mg. cells/ml. for the experiments done at pH 4, and 10–30 mg./ml. for those done at pH 6. Cells grown in pantothenate-deficient media tended to flocculate, and these were washed and resuspended in 0·01 M-phosphate buffer, pH 7.

The concentration of cell suspensions was estimated either by drying to constant weight at 100° or by measuring the turbidity with a Spekker absorptiometer, using neutral filters. The dry weight was related to the turbidity by means of an empirical calibration curve. All figures on cell concentrations given in this paper refer to dry weights.

Incubation of washed suspensions and measurement of gas changes. The experiments were carried out in conical Warburg manometer flasks of about 20, or, in some cases, 100 ml. capacity. A solution of pyruvate, together with the buffer and any other substrate, was placed in the main compartment and the unbuffered washed cell suspension in the side arm. Buffers were omitted from the cell suspensions because at the acid pH the enzymes concerned were inactivated on incubation without substrate. CO_2 output and O_2 uptake were measured manometrically by the method of Warburg & Yabusoe (1924). All gas changes are expressed in μl. and, for convenience of comparison, quantities of pyruvate, acetoin and glucose are also expressed in μl., 22·4 μl. being taken as equivalent to 1 μmol. Reaction rates are expressed in terms of *Q* values (μl. metabolite/mg. dry wt. cells/hr.).

Taking and preservation of samples. Suspensions were incubated at 25° (pH 6) or 37° (pH 4) until the rate of gas evolution indicated that the reaction had almost ceased or, in the case of slow reactions, until quantities of metabolites sufficient to be measured had accumulated. If acetoin alone was to be determined, samples were pipetted into 1 ml. 10 N-H_2SO_4 immediately after incubation. Samples for the estimation of pyruvic acid were acidified by the addition of 0·1 vol. 2 N-HCl from the side arm of the manometer, and were then incubated at 60° for 15 min. to inactivate the bacterial enzymes. Samples for the estimation of lactic acid or 2:3-butylene glycol were measured into the $CuSO_4$ solution used in the copper-lime precipitation, and stored in the ice chest. Samples for the estimation of volatile acid were treated with 4 ml. 1·3% (w/v) 2:4-dinitrophenylhydrazine in 2 N-H_2SO_4 to precipitate residual pyruvic acid (see Speck, Moulder & Evans, 1946). When acetaldehyde was estimated, the manometer cups, on removal from the bath, were stoppered and cooled in ice for 15 min.; the samples were then pipetted into the aeration tubes used in the determination.

Estimation of acetoin. Acetoin was oxidized to diacetyl according to Westerfeld (1945). After 15 min. treatment with the reagent, the solution was cooled and subjected to steam distillation in a micro-Kjeldahl apparatus. The diacetyl in 10 ml. distillate was estimated by the method of Eggleton, Elsden & Gough (1943) with a Spekker absorptiometer.

Estimation of lactic acid. Acetoin is not removed by treatment with copper-lime and, as it is volatile and bound by bisulphite, it interferes with the determination of lactic acid by the method of Friedemann & Graeser (1933). It is not sufficiently volatile to be removed by preliminary boiling, as is suggested by Friedemann & Graeser (1933) for the removal of other volatile bisulphite-binding substances. In order to remove the acetoin before estimation of lactic acid by the method of these authors, steam was passed through the solution in the Kjeldahl flask in which the determination was to be carried out. Distillation was continued for 15–20 min.,

until about 100 ml. water had been collected, the volume in the flask remaining approximately constant. These conditions were found to be sufficient for quantitative removal of the acetoin present.

Pyruvic acid was estimated by the carboxylase method (see Krebs & Eggleston, 1945).

Acetylcholine was assayed by means of the frog rectus abdominalis method (see Stephenson & Rowatt, 1947).

Volatile acid was estimated by the method of Friedemann (1938).

Acetaldehyde was transferred by a stream of air into a solution of bisulphite, as described by Rees (1946) for the estimation of threonine and serine, except that buffer was not added.

2:3-*Butylene glycol* was estimated by a method similar to that described by Johnson (1944), based on the oxidation of the substance to acetaldehyde by periodate. The solutions were precipitated with copper-lime and the filtrate treated with periodate without preliminary ether extraction. Acetoin and lactic acid are also oxidized to acetaldehyde by periodate. Acetoin was determined separately by the method given above; lactic acid was shown to be present in small amounts, about 5% of the pyruvate used. In calculating butylene glycol values, corrections were made for the acetoin and lactic acid present.

Succinate was estimated manometrically with succinic dehydrogenase (Krebs, 1937*b*).

Glucose was estimated by the fermentation method of Winzler (1944).

Preparation of pyruvic acid. Pyruvic acid was redistilled several times, immediately diluted with water to 2 N strength and stored at 4°. Before use the pH of the solution was adjusted to 4 by the addition of N-NaOH.

RESULTS

Products of pyruvate decomposition by Lactobacillus plantarum

Effect of pH *on products.* On addition of pyruvate to suspensions of *Lb. plantarum* a gas was evolved which was identified as CO_2 by the fact that it was absorbed by alkali. The rate of CO_2 formation with 0·008 M-pyruvate in citrate buffer (Kolthoff, 1932) with one suspension was 10 μl./mg. cells/hr. at pH 6, 113 at pH 5 and 272 at pH 4. At each of these pH values, acetoin was formed; the molecular ratio of CO_2 to acetoin was higher at pH 6 than at pH 5 or 4. Between pH 5·5 and 4 (citrate buffer) the ratio was 1·9; at pH 6·0 it varied between 4 and 6. The amount of CO_2 formed at pH 6·0 varied from 0·5 to 1 mol./mol. of pyruvate used, and the acetoin accounted for 20–32% of the pyruvate used, if it is assumed that 2 mol. of pyruvate yield 1 of acetoin and 2 of CO_2. At this pH, therefore, CO_2 and acetoin do not account for all the pyruvate metabolized. Volatile acid was formed which, calculated as acetic acid, accounted for about 6% of the pyruvate used. Lactic acid also accounted for about 6%. Acetaldehyde, 2:3-butylene glycol and succinate were not found.

At pH 4, the ratio of CO_2 to acetoin formed remained constant throughout the reaction (Fig. 1) and was approximately the same as that found by Silverman & Werkman (1941) in *A. aerogenes*. The rate of CO_2 formation was most rapid between pH 4·1 and 4·7 (Fig. 2). Further experiments on pyruvate metabolism were carried out at pH 4 for reasons to be stated below.

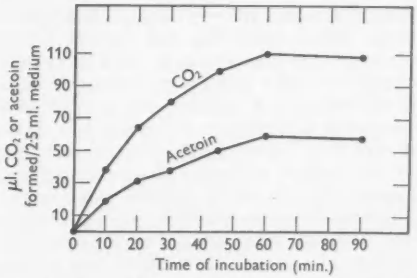

Fig. 1. Formation of acetoin and CO_2 from pyruvate. Cells grown in medium *P* with 10% boiled yeast extract (instead of 3% acid-treated yeast extract) for 16 hr. Cups contained 2·1 mg. cells, 0·02 M-citrate buffer (pH 3·5), 0·02 M-$KHPO_4^-$ and 280 μl. pyruvic acid (0·005 M) in 2·5 ml., 37°. Cups removed from the bath at 10 min. intervals for estimation of acetoin.

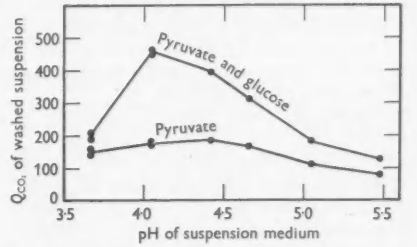

Fig. 2. Effect of pH on Q_{CO_2}. 1·4 mg. cells incubated in 2·5 ml. 0·02 M-citrate; 0·008 M-pyruvate; 0·004 M-glucose. 37°; N_2. Q_{CO_2} calc. for first 20 min.

Effect of oxygen. According to Silverman & Werkman (1941), the formation of acetoin from pyruvate by *Aerobacter* is independent of O_2. The rate of CO_2 formation from pyruvate by *Lb. plantarum* in citrate buffer was greater when O_2 was present than when N_2 was the gas phase, and more acetoin was formed, although the ratio of CO_2 to acetoin formed was more than 2 (2·8). In barbiturate buffer, more CO_2 was formed aerobically than anaerobically, but the quantity of acetoin formed was the same in each case. As the presence of O_2 appeared to complicate the reaction, experiments were carried out anaerobically.

Optical rotation and identification of acetoin formed. Hirsch (1922) obtained optically active acetoin from a fermentation of pyruvic acid in a culture of top yeast. The acetoin formed by yeast juice was

not optically active, and Dirscherl (1930) suggested that this was formed non-enzymically, only the decarboxylation of pyruvic acid to a 2-carbon unit being enzymic. The optical activity of the acetoin formed by *Lb. plantarum* was therefore tested.

A sufficient quantity of acetoin could be obtained only in the presence of glucose. It was therefore necessary to extract the acetoin from the suspension medium before measuring the optical activity. 1·62 g. cells were incubated at 37° for 5 hr. in 750 ml. 0·02 M-citrate buffer (pH 3·9) containing 25 ml. 2 M-pyruvate and 0·54 g. glucose. The suspension was concentrated according to the method of Fulmer, Underkoffer & Bantz (1943), clarified with kieselguhr, and further concentrated by saturation with anhydrous sodium sulphate at 40° and removal of the hydrated sulphate after cooling. The saturation of the solution with sodium sulphate facilitated extraction of acetoin. The solution was extracted with ether for 20 hr. in Kutscher-Steudel extractors. A few ml. water were added to the extract and the ether removed under reduced pressure. The fraction boiling at 29–30° at 19 mm. Hg pressure was collected. The 2:4-dinitrophenylosazone prepared from this fraction had a melting point of 317·5° (decomp.) (uncorr.); that of an authentic specimen of the osazone of acetoin was 317° (decomp.) (uncorr.). Of the substances present in the original solution which could be extracted by ether, pyruvic and citric acids are not optically active and lactic acid does not distil under the conditions used.

The distillate, when diluted to contain 62 mg. acetoin/ml., gave an $\alpha_D^{19°}$ of $-8·77°$ in a 2 dm. polarimeter tube. Thus $[\alpha]_D^{19°}$ was $-70·6°$. Silverman & Werkman (1941) report a value of $-100°$ for acetoin formed from pyruvate by *A. aerogenes*. In their experiments, as no other optically active substance was present in the fermentation liquor, the rotation was measured directly, with no attempt at purification.

Rate of decomposition of pyruvate by Lactobacillus plantarum

Effect of buffers. Citrate interferes with the determination of lactic acid by the method of Friedemann & Graeser (1933), and it was necessary to use another buffer when this estimation was to be carried out. In acetate buffer the rate of CO_2 formation was less than that found in citrate: in 0·04 M-acetate with 0·008 M-pyruvate the Q_{CO_2} was 84 % of that in 0·02 M-citrate; in 0·1 M-acetate it was 52 % and in 0·2 M 12 %. In 0·02, 0·06 and 0·1 M-citrate, the Q_{CO_2} values were, respectively, 243, 248 and 219. The rate of CO_2 formation from pyruvate in succinate buffer is shown in Table 1. At 0·04 M, the rate of the reaction was considerably lower than in citrate and was very little higher when the cells were incubated in a mixture of the two buffers. The effect, therefore, was not due to the absence of citrate. The addition of 0·008 M-succinate to cells incubated in citrate buffer did not affect the rate of CO_2 formation from pyruvate.

Table 1. *Inhibition by succinate of* CO_2 *formation from pyruvate by* Lactobacillus plantarum

(*Lb. plantarum*, 0·9 mg., incubated in 2·5 ml. solution containing 0·008 M-pyruvate and 0·002 M-glucose where added; citrate or succinate buffer pH 4 as stated.)

	Q_{CO_2} (10–20 min.)	
Addition	Pyruvate	Pyruvate and glucose
Succinate, 0·04 M	56	70
Citrate, 0·04 M	333	453
Succinate, 0·02 M, and citrate, 0·02 M	70	81
Citrate, 0·02 M	190	443
Citrate, 0·02 M, and succinate, 0·0008 M	203	427

When cells were incubated in 0·01 M-barbituric acid-NaOH buffer the rate of CO_2 formation from pyruvate and glucose (see below) was approximately the same as in citrate buffer; with pyruvate alone CO_2 formation was initially slower than in citrate buffer, but after 15 min. there was little difference between the two systems (Table 2). Consequently, as acetate would interfere with estimations of this substance, barbiturate was used as buffer when lactate was to be estimated and in some other experiments. It is noteworthy that no other buffer tested gave as high a rate of CO_2 formation as citrate. The Q_{CO_2} in 0·04 M-lactate with pyruvate was

Table 2. *Effect of barbiturate on rate of* CO_2 *formation from pyruvate by* Lactobacillus plantarum

(*Lb. plantarum*, 0·9 mg., incubated in 2·5 ml. citrate or barbituric acid-NaOH buffer with 0·008 M-pyruvate.)

	Q_{CO_2}			
Additions	5–10 min.	10–15 min.	15–20 min.	20–25 min.
Citrate, 0·02 M	180	202	202	157
Barbiturate, 0·02 M	67	67	67	67
Barbiturate, 0·01 M	66	131	153	88
Glucose, 0·002 M, and citrate, 0·02 M	577	533	512	422
Glucose, 0·002 M, and barbiturate, 0·02 M	133	333	488	355
Glucose, 0·002 M, and barbiturate, 0·01 M	402	594	764	529

13 % and that with pyruvate and glucose 3 % of that found in 0·02 M-citrate buffer.

Utilization of pyruvate. At 37°, the rate of CO_2 formation from pyruvate gradually decreased (Fig. 3). This fall in the rate of the reaction was not due to a change in pH. Although the conversion of 400 μl. pyruvate into acetoin may raise the pH from 4·0 to 4·7 in the presence of 0·02 M-citrate buffer, such a change in pH does not cause a major change in rate: at pH 4·7, the Q_{CO_2} was 160, and at pH 4·0 it was 175 (conditions as in Fig. 2). In later experiments, 0·06 M-citrate buffer was used, and, in this buffer, the pH did not change appreciably during the utilization of 400 μl. pyruvate.

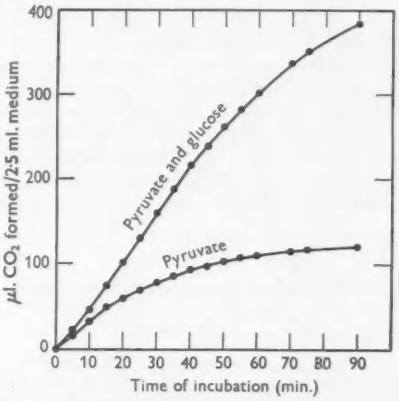

Fig. 3. Course of CO_2 formation from pyruvate. 0·34 mg. cells incubated at 37°, N_2, in 2·5 ml. 0·06 M-citrate buffer (pH 3·9); 0·008 M-pyruvate; 0·008 M-glucose.

The yield of acetoin and CO_2 accounted for all the pyruvate used at pH 4 (Table 3, Exp. *a*). When cells were incubated with a higher concentration of pyruvate, more CO_2 and acetoin were formed, but the quantity of pyruvate unused was greater than with the lower concentration of pyruvate. The decrease in the concentration of pyruvate, therefore, was not the factor stopping the reaction.

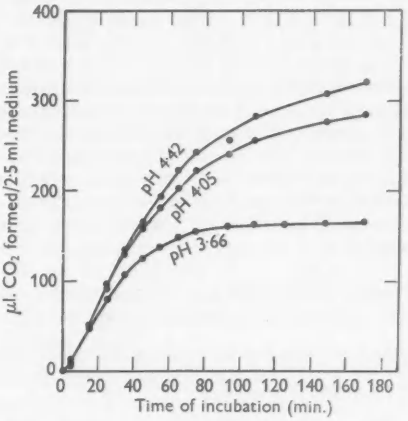

Fig. 4. Effect of pH on the course of CO_2 evolution. 1·4 mg. cells incubated in 2·5 ml. 0·02 M-citrate buffer at pH indicated; 0·008 M-pyruvate, 37°, N_2.

When different concentrations of the same suspensions of cells were incubated with the same concentration of pyruvate the final quantity of pyruvate utilized was approximately proportional to the weight of cells (Table 3, Exp. *b*). At 20° the rate of CO_2 formation from pyruvate was constant for 60 min. ($Q_{CO_2} = 113$). At 15° the rate of CO_2 formation by the same suspension was constant for 3·5 hr. ($Q_{CO_2} = 92$). The quantity of CO_2 formed by the cells

Table 3. *Factors influencing the metabolism of pyruvate at* pH 4

(Cells incubated in 2·5 ml. solution containing either 0·06 M-citrate, 0·01 M-barbiturate or 0·016 M-acetate.)

Exp.	Dry wt. of cells (mg.)	Buffer	Additions	Time (min.)	Temp. (°)	Initial pyruvate (μl.)	CO_2 formed (μl.)	Acetoin formed (μl.)	Residual pyruvate (μl.)	Recovery (%)
a	0·3	Citrate	—	190	37	0	10	—	—	—
						312	71	34	243	100
						547	159	88	400	113
b	0·35	Citrate	—	140	37	400	16	—	—	—
	1·9			140	37	400	69	—	—	—
c	1·8	Barbiturate	—	100	37	400	213	114	177	97
			Glucose (112 μl.)	100	37	400	345	190	38	96
d	0·9	Acetate	—	150	25	—	318	177	—	—
			Acetaldehyde (45 μl.)	150	25	—	259	133	—	—
			Glucose (224 μl.)	150	25	—	704	380	—	—
			Acetaldehyde + glucose	150	25	—	539	277	—	—

before the rate fell off was approximately the same in these two cases.

The rates of reaction fell off more rapidly at a lower pH (Fig. 4). The reason for this effect of pH was not discovered.

Effect of glucose. In the presence of glucose more pyruvate was decomposed than without glucose, but the acetoin and CO_2 formed again accounted for the pyruvate used (Table 3, Exp. *c*). There was no stoicheiometric relation between the glucose added and the effect produced, e.g. 20 μl. was sufficient to enable 0·4 mg. cells to decompose 300 μl. pyruvate. Glucose increased not only the quantity of pyruvate used, but also the rate of the reaction (Fig. 3). Using citrate buffer, the highest Q_{CO_2} values were obtained with 0·0004–0·001 M-glucose (Table 4). Glucose had no effect on the ratio CO_2 : acetoin formed.

Table 4. *Effect of glucose on* CO_2 *formation from pyruvate by* Lactobacillus plantarum

(2·5 ml. solution containing 1·6 mg. cells, 0·02 M-citrate buffer, pH 4·0, 0·008 M-pyruvate, incubated at 37° in N_2; Q_{CO_2} from 10th to 20th min.)

Glucose added		
(μl.)	(mM)	Q_{CO_2}
0	0	177
4·5	0·08	312
11·2	0·20	374
22·4	0·40	488
44·8	0·80	565

At pH 6·5 glucose is converted by the organism chiefly (80 %) to lactic acid (Stephenson & Rowatt, 1947). Lactic acid was also formed at pH 4 during the formation of acetoin from pyruvate in the presence of glucose. In an experiment in which 1·6 mg. cells formed 376 μl. CO_2 from 400 μl. pyruvate, 114 μl. lactic acid were formed from 114 μl. glucose and approximately 54 μl. glucose were recovered. In a parallel experiment, when glucose alone was added, neither glucose utilization nor lactate formation occurred. Barbiturate buffer was used in these experiments. The effect of glucose on the pH activity curve is shown in Fig. 2. Glucose sharpened the peak of the curve. The time course of the CO_2 evolution varied with the pH (Fig. 5). Adenosinetriphosphate (0·0016 M) reduced, and glutathione (0·04 % w/v) or sodium glycerophosphate (0·02 M) had no effect on the decomposition of pyruvate at pH 4.

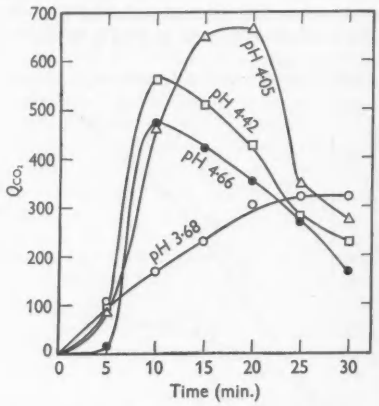

Fig. 5. Effect of pH on course of CO_2 formation from pyruvate in presence of glucose. 2·5 ml. solution containing 1·4 mg. cells, 0·02 M-citrate buffer, 0·008 M-pyruvate and 0·004 M-glucose incubated at 37° in N_2. The Q_{CO_2} recorded at each time is that obtaining over the previous 5 min.

The addition of phosphate to suspensions containing pyruvate alone did not affect the rate of CO_2 formation, but, in the presence of glucose and pyruvate, phosphate increased the maximum rate and delayed the fall in rate (Table 5). The quantity of acetoin formed was similarly increased.

Incubation of cells at 37° in citrate buffer at pH 3·9 without substrate reduced their ability to form acetoin and CO_2 from pyruvate at pH values below 4. At higher pH values little CO_2 was formed from pyruvate alone, but, in the presence of glucose and pyruvate, the rate of CO_2 formation by these pretreated cells gradually increased. The reactivation was very sensitive to pH; the Q_{CO_2} at pH 4·16 was, in the experiment shown in Table 6, twenty times greater than the Q_{CO_2} at pH 3·94.

Table 5. *Effect of phosphate on rate of* CO_2 *formation from pyruvate in presence of glucose by* Lactobacillus plantarum

(2·5 ml. solution containing 1·2 mg. cells, 0·04 M-citrate buffer (pH 4), 0·008 M-pyruvate incubated at 37° in N_2.)

Addition	Q_{CO_2} 5–15 min.	15–25 min.	25–35 min.	35–45 min.
None	240	124	54	—
0·02 M-Potassium phosphate, pH 4	252	133	86	—
0·008 M-Glucose	286	258	274	262
0·008 M-Glucose + 0·02 M-phosphate	313	346	522	417

Table 6. *Effect of glucose and* pH *on activity of pretreated cells*

(5 mg. cells incubated 20 min. at 37° in 12 ml. 0·02 M-citrate buffer (pH 3·8); centrifuged from medium, resuspended 0·9 % NaCl, incubated 0·06 M-citrate buffer. Initial pH estimated on buffer, final pH estimated on suspension medium.)

Substrate	Initial pH	Final pH	Q_{CO_2} 5–10 min.	Q_{CO_2} 30–35 min.
0·008 M-Pyruvate	4·16	4·17	123	25
0·008 M-Pyruvate + 0·008 M-glucose	3·94	3·90	100	10
	4·16	4·17	133	208

The pyruvate concentration used in the above experiments was not the optimal concentration; the rate of CO_2 formation increased with substrate concentration up to 0·01 or 0·03 M (Fig. 6) varying according to pH. Above this concentration the rate fell, and at 0·1 M very little CO_2 was formed. At this concentration the experimental error in the determination of pyruvate was too great to show whether

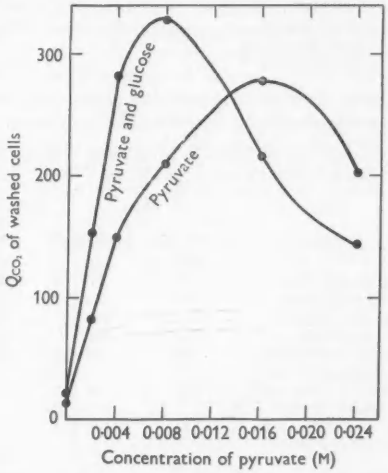

Fig. 6. Effect of substrate concentration on the rate of CO_2 formation from pyruvate. 1·6 mg. cells incubated in 2·5 ml. 0·06 M-citrate buffer (pH 4), 0·008 M-glucose, pyruvate concentration as stated. Q_{CO_2} with pyruvate from 5th to 10th minute; Q_{CO_2} pyruvate and glucose from 15th to 20th minute.

any pyruvate had been used. The rate of CO_2 formation from pyruvate in presence of glucose was similarly inhibited at high concentrations of pyruvate, but the inhibitory effect appeared at a lower concentration of pyruvate. High concentrations of pyruvate had the same effect on the formation of CO_2 from pyruvate by the strain of *A. aerogenes* used. With this organism, concentrations above 0·01 M were inhibitory.

Effect of inhibitors and acetaldehyde. Acetoin formation by cells incubated in citrate buffer with pyruvate was not inhibited by 0·001 M-cyanide. 0·004 M-Fluoride caused 61 % inhibition when glucose was absent and 84 % when it was present. 0·005 M-Azide inhibited the reaction by 97 % when glucose was absent and 95 % when it was present.

Acetaldehyde did not increase, but slightly inhibited, the formation of acetoin when it was added to cells incubated with pyruvate or with pyruvate and glucose (Table 3, Exp. *d*). The ratio of CO_2 to acetoin formed was virtually unaltered by acetaldehyde (1·8 when it was added and 1·9 when it was not).

Effect of dicarboxylic acids on the decomposition of pyruvate by Lactobacillus plantarum, Staphylococcus aureus *and* Aerobacter aerogenes

Effect of fumarate on Lb. plantarum. The reduced rate of CO_2 formation from pyruvate in the presence of succinate suggested that fumarate might be concerned in the breakdown of pyruvate. This compound, however, when added to suspensions of cells

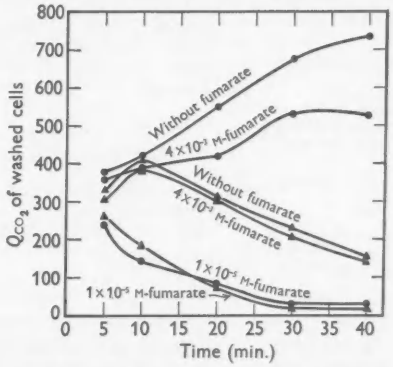

Fig. 7. The effect of varying concentrations of fumarate on the rate of CO_2 formation from pyruvate by *Lb. plantarum*. Cells (1·2 mg.) incubated in 2·5 ml. 0·06 M-citrate buffer (pH 4) containing (*a*) 0·016 M-pyruvate, ▲—▲; (*b*) 0·016 M-pyruvate + 0·008 M-glucose, ●—●.

incubated with pyruvate in citrate buffer, caused a strong inhibition of pyruvate utilization: for example, cells (1·1 mg.) incubated with 500 μl. pyruvate formed 454 μl. CO_2, while 459 μl. pyruvate were used; when 1120 μl. fumarate were added,

10 μl. CO_2 were formed and 20 μl. pyruvate used. The inhibition affected CO_2 and acetoin formation equally: 0·0025 M-fumarate inhibited CO_2 formation by 92 % and acetoin formation by 96 %; the figures with 0·0002 M-fumarate were 64 and 69 % respectively. The inhibition was progressive (Fig. 7) and reached over 90 % at 10^{-3} M-fumarate after 25 min. incubation. A significant inhibition was caused by only 1×10^{-4} M-fumarate. In the presence of glucose the degree of inhibition was greater.

Effect of fumarate on acetoin formation by Staph. aureus *and* A. aerogenes. In order to find whether fumarate generally inhibited this type of pyruvate breakdown, its effect on other organisms forming acetoin was tested. Lemoigne (1913) showed that Staphylococci formed acetoin when growing in media containing glucose. Washed cells of a strain of *Staph. aureus* were incubated with pyruvate under the conditions used for *Lb. plantarum* (pH 4·0). Acetoin and CO_2 were formed in the same proportions (2:1) as in *Lb. plantarum*, the Q_{CO_2} being about 900; the formation of each was inhibited by fumarate (Table 7). The inhibition was again progressive and reached 80 % in the presence of 0·005 M-fumarate after 11 min.

Table 7. *Inhibition by fumarate of formation of* CO_2 *and acetoin from pyruvate by* Staphylococcus aureus *and* Aerobacter aerogenes

(*Staph. aureus* (1·4 mg.) incubated 17 min. or *A. aerogenes* (0·8 mg.) incubated 20 min., each in 2·5 ml. 0·06 M-citrate (buffer pH 4).)

Additions	Pyruvate used (μl.)	CO_2 formed (μl.)	Acetoin formed (μl.)
Staph. aureus			
Pyruvate (0·008 M)	—	250	112
Fumarate (0·005 M)	—	40	1
Pyruvate (0·008 M) + Fumarate (0·005 M)	—	87	36
A. aerogenes			
Pyruvate (0·006 M)	240	260	81
Fumarate (0·005 M)	—	16	0
Pyruvate (0·006 M) + Fumarate (0·005 M)	67	71	24

Acetoin and CO_2 formation from pyruvate by *A. aerogenes* (see Silverman & Werkman, 1941) at pH 4 was also inhibited by fumarate (Table 7), but the concentration of fumarate required was higher than in the case of *Lb. plantarum*; only 30 % inhibition was caused by 0·004 M-fumarate. Further differences were that the ratio CO_2/acetoin exceeded 2 with some suspensions of this organism, and glucose did not increase the rate of CO_2 formation.

Effect of other acids on CO_2 *formation by* Lb. plantarum. Malate and maleate when added to suspensions of cells in citrate buffer caused approximately the same degree of inhibition as succinate; *cis-* and *trans*-aconitate and aspartate had a smaller effect (Table 8). Malonate at a concentration of 0·04 M caused 47 % inhibition. In this case the cells were incubated in acetate buffer (0·04 M) instead of citrate. All these compounds inhibited acetoin formation to approximately the same extent as CO_2 formation.

Table 8. *Effect of substances related to fumarate on* CO_2 *formation from pyruvate by* Lactobacillus plantarum

(Cells incubated with 0·06 M-citrate buffer, pH 4, 0·016 M-pyruvate. Inhibition measured as percentage drop in Q_{CO_2} after 20–25 min. incubation.)

Concentration of inhibitor ...	Inhibition (%) 0·004 M	0·015 M
Inhibitor		
Maleate	10	64
DL-Malate	16	69
cis-Aconitate	5	31
trans-Aconitate	0	15
DL-Aspartate	—	11

Table 9. *Inhibition by phthalate of* CO_2 *formation from pyruvate by* Lactobacillus plantarum

(Cells (1·9 mg.) incubated in 2·5 ml. 0·06 M-citrate buffer (pH 4) with 0·016 M-pyruvate for 130 min.)

Additions	Q_{CO_2} 5–10 min.	Q_{CO_2} 20–25 min.	Total CO_2 formed (μl.)
None	208	174	362
Phthalate, 0·004 M	39	20	37
Phthalate, 0·002 M	119	89	153
Phthalate, 0·001 M	149	129	217
Glucose, 0·008 M	231	508	782
Glucose, 0·008 M, and phthalate, 0·004 M	40	25	46
Glucose, 0·008 M, and phthalate, 0·002 M	113	89	177
Glucose, 0·008 M, and phthalate, 0·001 M	135	153	705

With the exception of fumarate, phthalate had the highest inhibitory activity of the substances tested. The inhibition of Q_{CO_2} by 0·001 M-phthalate was 30 % in the presence of pyruvate alone and 70 % in the presence of pyruvate and glucose (Table 9). At the two higher phthalate concentrations shown in this table, the rate of CO_2 formation was the same whether glucose was added or not. Phthalate did not alter the ratio CO_2:acetoin formed. The inhibition by lactate and acetate, and especially succinate and barbiturate, may be of the same type as those mentioned in this section.

Effect of aneurin on pyruvate decomposition by Lactobacillus plantarum

Comparison of normal and aneurin-deficient cells. That cocarboxylase is required for the formation of acetoin and CO_2 from pyruvate has been shown by

Table 10. *Effect of aneurin*

(Cells grown in medium *P* without yeast extract, with or without 0·1 μg. aneurin/ml. Cells incubated in 2·5 ml. 0·06 M-citrate pH 4·1, 0·008 M-pyruvate, at 37° in N_2.)

Additions	Aneurin-deficient cells (0·6 mg.) Q_{CO_2} 15–20 min.	Aneurin-deficient cells (0·6 mg.) CO_2/acetoin	Aneurin-rich cells (0·7 mg.) Q_{CO_2} 15–20 min.	Aneurin-rich cells (0·7 mg.) CO_2/acetoin
None	55	—	166	2·5
0·008 M-Glucose	96	4	554	1·9
Aneurin, 8 μg./ml.	47	—	180	2·1
Glucose + aneurin	664	1·9	567	2·1

Silverman & Werkman (1941) with *A. aerogenes* and by Green, Westerfeld, Vennesland & Knox (1942) with heart muscle. When *Lb. plantarum* was grown in a medium deficient in aneurin, the crop of cells was little less than in the presence of aneurin (e.g. 5·6 and 4·8 mg./100 ml.), but the deficient cells, when incubated with pyruvate, or pyruvate and glucose, formed very little CO_2 or acetoin. Addition of aneurin alone did not increase the CO_2 formation from pyruvate, but on incubation with pyruvate, glucose and aneurin, deficient cells formed CO_2 and acetoin at the same rate as cells grown in a medium containing aneurin. The quantity of CO_2 formed by the deficient cells was 1 mol./mol. of pyruvate used, whether aneurin was added or not. Aneurin did not affect the rate of CO_2 evolution by cells grown in a normal medium (Table 10).

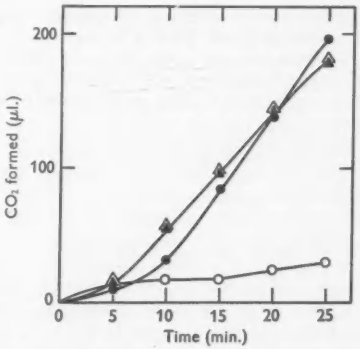

Fig. 8. The effect of aneurin on the course of CO_2 formation from pyruvate in presence of glucose. Conditions as in Table 10. Cells grown in aneurin-deficient medium (1) incubated with glucose, ○—○; (2) incubated with glucose + aneurin, ●—●. Cells grown in aneurin-rich medium (1) incubated with glucose, △—△; (2) incubated with glucose + aneurin ▲—▲.

In the experiment shown in Table 10 the time taken by normal cells to reach the maximum rate of CO_2 formation with glucose was the same as that taken by aneurin-deficient cells to which aneurin and glucose had been added (Fig. 8).

Effect of pantothenate on metabolism of pyruvate by Lactobacillus plantarum

Cells grown in pantothenate-deficient medium, when incubated at pH 4, formed acetoin at the normal rate, and this was not affected by the addition of pantothenate (Table 11). The same cells formed little acetylcholine unless pantothenate was added to the suspension medium. Nor had pantothenate a definite effect on the rate of pyruvate decomposition at pH 6, nor on the O_2 uptake.

Table 11. *Effect of pantothenate on the formation of acetylcholine and acetoin by pantothenate-deficient cells of* Lactobacillus plantarum

(Cells grown in 1 l. medium without pantothenate 20 hr., washed and made up in 0·01 M-phosphate buffer (pH 7). Acetylcholine formation tested in suspensions containing 1 mg. cells in 5 ml. 0·3 M-acetate buffer (pH 6·5), 0·001 M-glucose, 0·05% choline HCl; 210 min. incubation at 25° in N_2. Acetoin formation tested in suspensions containing 0·6 mg. cells in 2·5 ml. 0·02 M-citrate buffer (pH 4), 0·01 M-glucose, 0·04 M-pyruvate; 120 min. incubation. Pantothenate concentrations 0·16–0·2 μg./ml.)

Metabolite formed	Control	Calcium pantothenate added
Acetylcholine (μg. in 5 ml.)	0·6	12·2
Acetoin (μl. in 2·5 ml.)	123	128
Q_{CO_2} (0·004 M-pyruvate)	190	240
Q_{CO_2} (0·004 M-pyruvate + 0·01 M-glucose)	330	342

DISCUSSION

A quantitative formation of acetoin from pyruvate has previously been found in *Aerobacter* by Silverman & Werkman (1941), but has not been described in *Lb. plantarum* and related organisms, although small quantities of this compound are formed during the growth of many bacteria (Stahly & Werkman, 1942). The following four factors were found to affect the rate of acetoin formation from pyruvate: pH; glucose in catalytic quantities; aneurin; phosphate. The effects of aneurin and phosphate were observed by previous workers in other organisms. Pantothenate, which is required by the organism for the synthesis of acetylcholine, is not necessary for the formation of acetoin.

The mechanism of acetoin formation is not fully understood, and at the present stage of knowledge no explanation of the glucose effect can be offered. The fact that the acetoin formed is optically active indicates that it is formed by an enzymic process (see Dirscherl, 1930).

No explanation can be offered for the fact that low concentrations of fumarate inhibit the formation of acetoin from pyruvate by *Lb. plantarum*, *A. aerogenes* and *Staph. aureus*. It is noteworthy that in *Staph. aureus* at pH 7·0, when pyruvate is broken down by a different route, the anaerobic decomposition of pyruvate is stimulated by fumarate (Krebs, 1937*a*). The effects of other dicarboxylic acids such as phthalate, malate, maleate, malonate and succinate are probably based on the same type of reaction as the inhibition by fumarate.

The formation of acetoin from pyruvate is one of the few relatively rapid reactions known to occur in *Lb. plantarum*.

SUMMARY

1. *Lactobacillus plantarum* converted pyruvate quantitatively into laevorotatory acetoin and carbon dioxide at pH 4. At pH 6 pyruvate decomposition was slower and the amount of pyruvate was greater than the sum of acetoin, carbon dioxide, lactic and acetic acids formed.

2. The initial rate of carbon dioxide formation from pyruvate gradually fell, especially when the pH was below 4. Addition of glucose in catalytic amounts raised the Q_{CO_2} and delayed its fall.

3. Potassium phosphate further increased the Q_{CO_2} and delayed its fall in the presence of glucose but not in its absence.

4. At high concentrations of pyruvate (0·1 M), carbon dioxide formation was strongly inhibited.

5. Preliminary incubation of cells at pH 3·8 in buffer solutions without substrate reduced the capacity of the cells to decompose pyruvate. At pH values above 4 the capacity could be restored by glucose.

6. The decomposition of pyruvate by *Lactobacillus plantarum* at pH 4 is inhibited by low concentrations of fumarate. At all concentrations of fumarate, the degree of inhibition of acetoin and carbon dioxide formation is approximately the same. The degree of inhibition by a given concentration of fumarate is greater when glucose is present than when it is not.

7. The formation of carbon dioxide and acetoin from pyruvate by strains of *Aerobacter aerogenes* and *Staphylococcus aureus* is also inhibited by fumarate, but with these organisms a higher concentration of fumarate is required.

8. The formation of carbon dioxide and acetoin by *Lactobacillus plantarum* is inhibited to a lesser degree by phthalate, succinate, DL-malate, maleate, malonate, barbiturate, *cis*- and *trans*-aconitate and DL-aspartate.

9. The concentration of pantothenate in the suspension medium did not affect the rate of pyruvate decomposition.

10. Cells grown in a medium deficient in aneurin formed very little carbon dioxide or acetoin unless both aneurin and glucose were added to the suspension.

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The Amino-acid Sequence in the Phenylalanyl Chain of Insulin

1. THE IDENTIFICATION OF LOWER PEPTIDES FROM PARTIAL HYDROLYSATES

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When insulin is oxidized with performic acid, the —S—S— bridges of the cystine residues are broken by conversion to —SO_3H groups (Sanger, 1949*a*) and the molecule is split into its separate polypeptide chains. From the oxidized insulin two fractions could be isolated: an acidic fraction *A*, which contained only glycyl *N*-terminal residues (see below) and no basic amino-acids, and a basic fraction *B*, having phenylalanyl *N*-terminal residues. These appeared to be the only significant fractions present.

From a study of the partial hydrolysis products of the dinitrophenyl (DNP) derivatives of the two fractions it was possible to determine the sequence of the amino-acids adjoining the *N*-terminal residues and adjoining the lysine residues (Sanger, 1949*b*). In the case of fraction *B* the terminal sequence was shown to be Phe.Val.Asp.Glu and the lysine residues were present in the sequence Thr.Pro.-Lys.Ala (abbreviations for amino-acids are given in Table 1 below). No DNP-peptides were present which did not fit into these sequences, and from an estimation of the yields of the DNP-peptides produced on partial hydrolysis of DNP-insulin it was concluded that all the *N*-terminal phenylalanyl residues of insulin and all the lysine residues were present in the above two sequences respectively, and hence that there was only one type of phenylalanyl polypeptide chain in insulin. Similar though rather less clear-cut results were obtained for fraction *A*. Assuming a molecular weight of 12,000, it was concluded from these experiments that insulin is built up of two identical phenylalanyl polypeptide chains and two identical glycyl chains, these four chains being joined together by six —S—S— bridges. Various possible structures for the molecule were suggested (Sanger, 1949*c*).

The results with the terminal peptides gave a preliminary indication that the fractions *A* and *B* are both relatively homogeneous preparations of molecules having a single polypeptide chain and containing approximately 20 and 30 amino-acid residues respectively. It thus seemed that an investigation of the smaller peptides produced on partial hydrolysis might yield considerable information about the overall amino-acid sequence in these fractions. Consden, Gordon & Martin (1947) have described a method for the fractionation of lower peptides using paper chromatography which was successfully used to determine the pentapeptide sequence of 'gramicidin S' (Consden, Gordon, Martin & Synge, 1947). The present paper describes the application of this technique to partial acid hydrolysates of fraction *B* and the determination of a number of amino-acid sequences.

Throughout this paper the abbreviations for the amino-acid residues suggested by Brand & Edsall (1947) are used. These are listed in Table 1. In referring to peptides of known structure, the abbreviations for the residues are joined by a full stop (e.g. glycylalanine is written Gly.Ala). When two or more residues are included in square brackets the order is unknown. Thus, for instance, Gly.-[Ala, Leu] refers to a peptide or peptides containing glycine, alanine and leucine, in which the free amino group is on the glycine residue, but the relative order of the alanine and leucine residues is unknown. The residues having the free α-amino groups in peptides will be referred to as *N*-terminal residues, those with free α-carboxyl groups as *C*-terminal residues.

Table 1. *Abbreviations for amino-acid residues*

Amino-acid	Abbreviation
Cysteic acid	$CySO_3H$
Aspartic acid	Asp
Glutamic acid	Glu
Serine	Ser
Glycine	Gly
Threonine	Thr
Alanine	Ala
Tyrosine	Tyr
Valine	Val
Leucine	Leu
Phenylalanine	Phe
Proline	Pro
Histidine	His
Lysine	Lys
Arginine	Arg
Ornithine	Orn

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METHODS

Fraction *B* was prepared from crystalline ox insulin as previously described (Sanger, 1949*a*).

Hydrolysis and preliminary group separations

Since the partial hydrolysates were too complex for direct fractionation by paper chromatography, it was necessary to carry out certain preliminary group separations to divide them into a number of simpler fractions containing fewer peptides. For this purpose it is advantageous to use methods depending on physical properties of the peptides different from those responsible for the separations on paper chromatograms. We have used adsorption on charcoal, separation on synthetic ion-exchange resins and ionophoretic methods using three- or four-compartment cells. Ionophoresis in silica jelly, which has been successfully used by Consden, Gordon & Martin (1949) to fractionate acidic peptides, was not used since difficulties are encountered with peptides containing basic or aromatic amino-acids, both of which are present in fraction *B*.

hydrolysate was then transferred to a column of Amberlite IR-4B (British Drug Houses Ltd.) which had been equilibrated to pH 3·3, as described by Consden, Gordon & Martin (1948). The column, which was 10 cm. high and had a diameter of 1 cm., was washed with 200 ml. dil. HCl at pH 3·3, and the effluent, which contained neutral and basic peptides, was taken to dryness. The acidic peptides were eluted from the column with 100 ml. N-HCl and the solution taken to dryness and refractionated on a column of Amberlite IR-4B (10 cm. high) which had been equilibrated to pH 2·6. This column was developed with dil. HCl at pH 2·6. The first 100 ml. of effluent contained mainly peptides of aspartic and glutamic acids and was referred to as fraction $B1\beta$; the peptides of cysteic acid were collected in the next 200 ml. of effluent, which was fraction $B1\alpha$. A further fraction could be eluted from the column with N-HCl. This contained chiefly free cysteic acid together with a few peptides.

The mixture of neutral and basic peptides obtained from the Amberlite-pH 3·3 column was dissolved in a small volume of 5% (w/v) aqueous acetic acid and transferred to a column of charcoal (activated charcoal B.D.H. previously washed with 20% acetic acid), of diameter 1 cm. and height

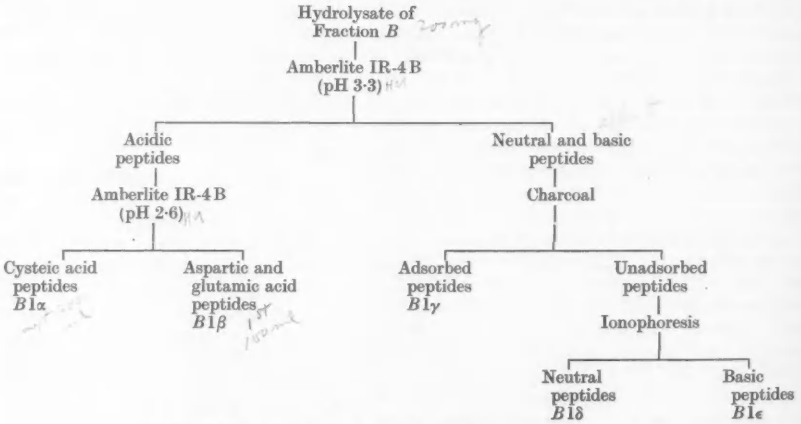

Fig. 1. Preliminary group separations in Exp. *B*1.

A number of experiments have been carried out using different methods both with the object of obtaining different peptides and also to investigate the various techniques. Three such experiments with hydrolysates made with 11 N-HCl are described below. Probably none of them represents the most satisfactory method of fractionating the mixture, but they do indicate the types of separations that can be obtained. The first experiment may be regarded as a preliminary one and the others, in which fewer fractions were taken, were largely confirmatory. Since sufficient peptides were not obtained from the 11 N-HCl hydrolysates to work out the entire sequence of amino-acids for fraction *B*, other methods of hydrolysis were studied. Experiments employing dilute acid (*B*4) and alkali (*B*5) for hydrolysis are also described.

*Experiment B*1. Fig. 1 summarizes the various stages of the fractionation used in this experiment. Fraction *B* (200 mg.) was hydrolysed for 4 days at 37° in 11 N-HCl, and excess HCl was removed by evaporation *in vacuo*. The

10 cm., which was washed with 150 ml. 5% acetic acid. The effluent containing unadsorbed peptides was collected and taken to dryness. A further adsorbed fraction ($B1\gamma$) was obtained by washing the charcoal with a solution of 5% (w/v) phenol in 20% (w/v) aqueous acetic acid. The object of this treatment was to separate a fraction containing peptides of aromatic amino-acids (Tiselius, Drake & Hagdahl, 1947). In fact most of these peptides had already been lost by adsorption on the Amberlite, and this fraction contained other more strongly adsorbed peptides. Nevertheless, quite a valuable fractionation was obtained. The unadsorbed fraction was finally separated into a neutral ($B1\delta$) and a basic ($B1\epsilon$) fraction by ionophoresis in a simple three-compartment cell of the type used by Macpherson (1946).

*Experiment B*2. In this experiment we simply separated the hydrolysate into three fractions using a four-compartment ionophoresis cell that was designed by Dr R. L. M. Synge (1951) and which he kindly described to us. This is essentially the cell of Theorell & Åkeson (1942), to

which is added an extra anode compartment containing H_2SO_4 as in the desalting apparatus described by Consden, Gordon & Martin (1947). In this way the acid fraction is prevented from coming into contact with the anode, where decompositions are likely to take place, especially if chlorine is being evolved.

Fig. 2 shows a diagram of the cell as used in the present experiment. It differs only in certain details from that of Synge. Membrane *a* is of cellophan, and membranes *b* and *c* of formolized gelatin (Synge has membrane *b* of formolized sheep-skin parchment and *c* of cellophan). The cell was connected to 200 V. d.c. mains with a 40 W. filament lamp in series and was cooled during the run.

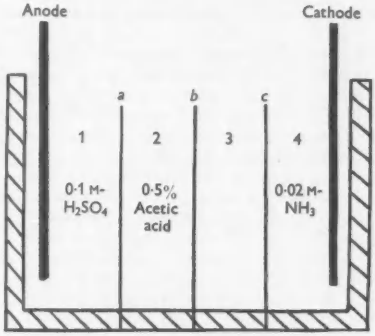

Fig. 2. Diagram of four-compartment ionophoresis cell. (For explanation see text.)

Fraction *B* (70 mg.) was hydrolysed 3 days at 37° in 11 N-HCl. After removal of excess HCl, the hydrolysate was adjusted to pH 5 and put in compartment 3 of the above cell. On applying the voltage an initial current of 110 ma. was produced and this rose to a maximum of 170 ma. and then slowly dropped. The pH of compartment 3 (which was stirred during the run) was maintained at about pH 4·5 (green to bromocresol green) by the addition of NH_3. It is necessary to add considerable amounts of NH_3 which leads to an increase in the pH of compartment 4, which may prevent the weakly basic histidine peptides from entering this compartment. It is therefore necessary to change the aqueous NH_3 solution in compartment 4 twice during the run. This also prevents some decomposition of arginine peptides, which seems to occur on prolonged ionophoresis. The separation was continued for about 1 hr. when the current had dropped to 25–30 ma. The contents of compartments 2, 3 and 4 were collected and taken to dryness. The acidic fraction from compartment 2 is referred to as $B2\alpha$, the neutral one from compartment 3 as $B2\beta$ and the basic one from compartment 4 as $B2\gamma$.

*Experiment B*3. Fraction *B* (113 mg.) was hydrolysed in 11 N-HCl at 37° for 3 days. After removal of the HCl *in vacuo*, the residue was dissolved in 5 ml. water and shaken with 100 mg. charcoal for 20 min. The charcoal was filtered off and washed with 10 ml. 5% (w/v) aqueous acetic acid. The filtrate was then fractionated into acidic ($B3\alpha$), neutral ($B3\beta$) and basic ($B3\gamma$) fractions in the four-compartment cell as described in Exp. *B*2. A further fraction ($B3\delta$) was eluted from the charcoal with 20 ml. 5% phenol in 20% acetic acid.

*Experiment B*4. Fraction *B* (50 mg.) was boiled for 24 hr. under reflux with 0·1 N-HCl (50 ml.). After removal of the HCl *in vacuo* the residue was subjected to ionophoresis as described in Exp. *B*2, and acidic ($B4\alpha$), neutral ($B4\beta$) and basic ($B4\gamma$) fractions were obtained.

*Experiment B*5. Fraction *B* (40 mg.) was heated at 105° in a sealed tube for 5 hr. with 5 ml. 0·2 N-NaOH. After neutralization with H_2SO_4 the mixture was desalted according to Consden, Gordon & Martin (1947). It was then subjected to ionophoresis as above.

Paper chromatography of peptides

Samples (corresponding to about 10 mg. of the original fraction *B*) of the various fractions obtained after group separations were subjected to two-dimensional chromatography on Whatman no. 4 filter paper. The various solvent systems used are described below:

Phenol-0·3% NH_3. 0·3% (w/v) Aqueous NH_3 (1 l.) was put in the bottom of the chromatography tank. Phenol saturated with water was used in the trough, and the tank was filled with coal gas.

Cresol-0·03% NH_3. As above, but containing 1 l. 0·03% (w/v) aqueous NH_3 and developed with *m*-cresol.

Collidine. The collidine used in this work gave considerably lower R_F values for the amino-acids than that used by Consden, Gordon & Martin (1944, see Dent, 1948).

Butanol-acetic acid. Prepared according to Partridge (1948) by mixing 5 vol. water, 4 vol. *n*-butanol and 1 vol. acetic acid. The mixture was usually kept a few days before use, and as no special precautions were taken to ensure that all samples were equally esterified the R_F values were not always constant and consequently have not been recorded. However, in Figs. 5–16, the distances of the peptide spots from the origin are given.

Other solvents used were prepared as described by Consden *et al.* (1944).

As a rule the chromatograms were developed first with phenol or cresol in the long direction of the paper and in the second direction with another solvent (usually butanol-acetic acid). If phenol was used as the second solvent, the fluorescence of the dried spots could not be seen. For certain slow-moving peptides it was necessary to run the solvent considerably farther than the end of the filter paper. This was accomplished by attaching a second folded sheet of filter paper to the front of the chromatogram. Where this was done the number of lengths of filter paper through which the solvent ran is indicated in Figs. 5–16. After development, the chromatograms were dried, heated at 100° for 15–30 min. to bring out the fluorescence of the spots (Phillips, 1948) and examined under the ultraviolet lamp. The fluorescent areas were marked on the paper. As observed by Jones (1949), the fluorescence was found to depend to a certain extent on the particular batch of filter paper used. In order to obtain the maximum effect it was necessary to avoid any contact of the papers with phenolic vapours as, for instance, in the drier. Where good fluorescent spots were seen they could be cut out directly without treatment with ninhydrin. Usually at least one replicate sheet was treated with ninhydrin to locate the peptides, and this could be used as a reference for cutting out the other sheets on which fluorescent spots were marked. 0·025% (w/v) Ninhydrin in water-saturated *n*-butanol was used to spray the sheets. This gave a sufficient colour development with very little destruction of the peptide, so that the eluates from the ninhydrin-treated spots could be used to

identify the amino-acids produced on hydrolysis. In certain cases, depending on the amino-acids concerned, there appeared to be a slight destruction of the *N*-terminal residue, but this was usually negligible. Where the peptide was to be deaminated or treated with fluorodinitrobenzene (FDNB), the use of ninhydrin was avoided and eluates from spots cut from untreated sheets were used. It was found that, if eluates from spots which had been treated with 0·025% ninhydrin were subjected to deamination, all the amino-acids present were almost completely destroyed.

Elution of peptides

For the elution of the peptides from the paper a combination of the methods of Consden, Gordon & Martin (1947) and of Dent (1947) was found convenient.

The spots were cut out and the cut trimmed to a point at one end. The other end was held between two microscope slides, which were placed in a trough of water as shown in Fig. 3. The solution passed into a capillary tube which was

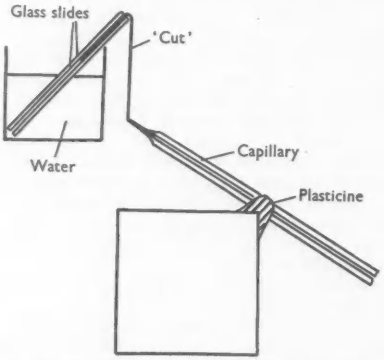

Fig. 3. Method of eluting peptides from filter paper.

held on a wooden block by a piece of plasticine. When sufficient liquid had collected, the contents were transferred to a polythene strip and dried *in vacuo*.

Identification of amino-acid residues present in peptides

Hydrolysis of the peptides was carried out with 5·7 N-HCl in capillary tubes as described by Consden, Gordon & Martin (1947), and the resulting amino-acids identified by paper chromatography. Fraction *B* contains the amino-acids listed in Table 14. It was shown by chromatography with benzyl alcohol that isoleucine is absent.

On account of the limited chromatographic apparatus available, and the labour involved, it was desirable to use one-dimensional chromatograms as far as possible to identify the amino-acids present. In the earlier experiments phenol-0·3% NH_3 was used for the preliminary identification of all peptide hydrolysates. Cysteic acid, aspartic acid, glutamic acid, serine, glycine, threonine and alanine can all be unequivocally identified in this solvent. The other faster moving amino-acids cannot be so easily distinguished especially if several of them are present. They may be more satisfactorily identified if phenol-0·03% NH_3 is used. The basic amino-acids move slower on this solvent and may frequently be recognized. The identification of all these faster moving amino-acids was always confirmed using some other solvent system or on a two-dimensional chromatogram.

For the complete analysis of a peptide two-dimensional chromatograms were run on half sheets (46 × 28 cm.) of Whatman no. 1 filter paper, which were developed in the long direction with phenol-0·3% NH_3 and in the short with butanol-acetic acid. All the amino-acids present in fraction *B* may be recognized on such a chromatogram (Fig. 4*b*).

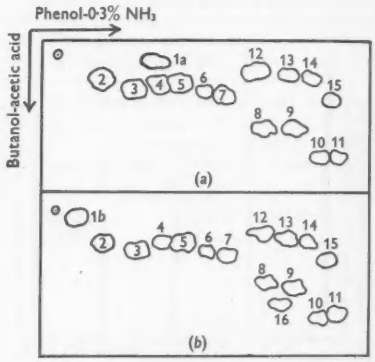

Fig. 4. Chromatograms of hydrolysates of insulin (*a*) and fraction *B* (*b*) run on half sheets of Whatman no. 1 filter paper. The identities of the spots are as follows: 1*a*, cystine; 1*b*, cysteic acid; 2, aspartic acid; 3, glutamic acid; 4, serine; 5, glycine; 6, threonine; 7, alanine; 8, tyrosine; 9, valine; 10, leucine; 11, phenylalanine; 12, histidine; 13, lysine; 14, arginine; 15, proline; 16, TyrX.

On hydrolysis of certain aromatic peptides a new spot was observed moving slightly faster than tyrosine in phenol and almost as fast as leucine in butanol-acetic acid. It gave a positive reaction with diazotized sulphanilic acid. In many cases pairs of peptides were found, one containing tyrosine and the other this new amino-acid. It is thus considered to be a breakdown product of tyrosine produced during the oxidation with performic acid, and will be referred to as TyrX. Fig. 4*b* shows a chromatogram of a hydrolysate of fraction *B* and Fig. 4*a* one of a hydrolysate of insulin. The TyrX spot (16) can be seen only in Fig. 4*b* below the tyrosine and valine spots, thus showing that it has been formed during the preparation of fraction *B*.

Since histidine gives a relatively weak spot with ninhydrin, it is possible to overlook it in certain peptides. The following modification of the Pauli test which avoids the use of aqueous solutions was found to be suitable for the identification of histidine and histidine peptides on chromatograms.

A mixture of equal volumes of 1% (w/v) *p*-anisidine in ethanolic 0·11 N-HCl and 10% (v/v) amyl nitrite in ethanol was allowed to stand a few minutes and then sprayed on to the paper. The sheets were dried in air and the colour developed with NH_3 vapour or by spraying on ethanolic KOH solution. Histidine and histidine peptides give a dark-red spot, whereas tyrosine gives a faint orange colour.

Determination of the structure of peptides

Deamination. In order to determine the *N*-terminal residues of the peptides we have deaminated them with nitrosyl chloride as described by Consden, Gordon & Martin (1947). In some experiments the reaction was carried out at 37° for 10 min., in others at room temperature for 30 min. No difference was noticed between the two procedures. For most peptides the method gave satisfactory results, though it was rarely possible to destroy the *N*-terminal residue completely. Certain amino-acids such as leucine and glutamic acid seemed to be especially resistant to this treatment, and if these were present as *N*-terminal residues, clear-cut deamination could rarely be obtained. Results with peptides containing tyrosine or arginine were also difficult to interpret.

DNP method. For most of the peptides the *N*-terminal residue has also been determined by the DNP technique, which usually gives clear-cut results, though it is rather more laborious. In some cases the DNP-derivative of the terminal residue was identified directly by chromatography using the micro method previously described (Sanger, 1949*b*). As this requires rather more material than is usually available from a paper chromatogram, it is often convenient to use an alternative procedure, and to identify the amino-acids present after hydrolysis of the DNP-peptide. Although traces of free amino-acids are formed on hydrolysis of DNP amino-acids (Consden, Gordon, Martin & Synge, 1947), it was found that they are sufficiently stable to ensure that the terminal residue is almost completely absent. The results are usually much more clear-cut than when the deamination method is used.

In this method the peptide is treated with FDNB in the usual way and the excess reagent removed by extraction of the alkaline solution with ether. It is then necessary to separate the DNP-peptide from the salt present. This may be done by extracting it from acid solution into ethyl acetate, or if it cannot be extracted by adsorbing it on a talc column from acid or neutral solution (Sanger, 1949*b*). The DNP peptide is then hydrolysed for 20 hr. with 5·7 N-HCl at 105° and the amino-acids remaining identified. In the case of peptides containing glycine as *N*-terminal residue a certain amount of breakdown of the DNP-glycine to free glycine was observed so that the results were not so clear. Better results were obtained if a shorter time of hydrolysis was used. Lysine, tyrosine and histidine, if present in a peptide with their amino groups blocked, are converted to their monoDNP derivatives which can be identified on paper chromatograms. Table 2 shows R_F values for these with butanol-acetic acid which had been prepared 24 hr. before use. The values for α-*N*-DNP-arginine, DNP-cysteic acid and for valine and leucine are given for comparison. Although *O*-DNP-tyrosine and *im*-DNP-histidine are colourless, they show up as dark spots if the papers are examined in ultraviolet light. This effect would seem to be due to quenching the fluorescence of the paper. Peptides containing *im*-DNP-histidine may be successfully fractionated on paper chromatograms with solvents such as collidine and pentanol.

RESULTS

The results obtained with the various fractions are given in Figs. 5–16 and Tables 3–13.

Figs. 5–16 are diagrams of the two-dimensional chromatograms, from which the peptide spots were eluted, and Tables 3–13 give the results obtained with the eluted peptides. In column 2 of these tables are listed the amino-acids identified on complete hydrolysis, and the approximate strength is indicated in column 3. The × 's have the same significance as in the paper of Consden *et al.* (1949), and ? represents a very weak spot. Column 4 shows the strength of the amino-acids remaining after deamination with NOCl and hydrolysis of the peptide and column 5 that remaining after hydrolysis of the DNP peptide. In the last column are given the structures of the peptides, as far as they can be deduced from the data given in the tables. As far as possible all the results with one peptide are given together, although they were often obtained in different experiments, and sometimes from the same peptide in a different fraction. Most of the results recorded have been obtained in several experiments. Where deamination or the DNP method has been used only the most satisfactory and clear-cut results have been recorded. While such a procedure does not necessarily give a true indication of the efficiencies of the methods it is probably justified, as much more can be learnt from such a result than from an indefinite one. This is especially true for the deamination technique where several experiments were usually carried out on each peptide.

Besides the peptides shown in Fig. 5 and Table 3, fraction *B*1α contained traces of $CySO_3H$.Ala and Ser.Val.$CySO_3H$. These peptides were probably derived from the small amount of fraction *A* contained in the preparation of fraction *B*, since they were found in very high concentration in fraction *A*. The presence of Ser.Val.$CySO_3H$ is suggested by a trace of serine that was sometimes noticed in spot 3. With this fraction there was frequently overlapping between spots 3 and 4 and between spots 5 and 6. When the eluate from the Amberlite-pH 2·6 column was collected in several successive fractions, a slower moving fraction was obtained which contained peptides 4 and 6 without the corresponding peptides containing glycine. The smaller peptides are retained more strongly on the resin than the larger.

Two chromatograms of fraction *B*1γ are illustrated in Figs. 7 and 8, since certain peptides are separated better using cresol and others with phenol. The combined results are given in Table 5.

Table 2. *R_F values of water-soluble DNP derivatives*

(Whatman no. 4 filter paper; butanol-acetic acid.)

	R_F	Colour of spot	Ninhydrin reaction
O-DNP-Tyrosine	0·84	Colourless	Purple
α-*N*-DNP-Arginine	0·81	Yellow	No reaction
ε-*N*-DNP-Lysine	0·77	Yellow	Brown
Leucine	0·67	—	—
im-DNP-Histidine	0·57	Faint yellow	Brown
Valine	0·49	—	—
DNP-Cysteic acid	0·42	Yellow	—

The results with fraction $B1\epsilon$ are recorded with the results on fraction $B2\gamma$, which gave a more complete mixture of basic peptides. $B1\epsilon$ was identical with $B2\gamma$ except that

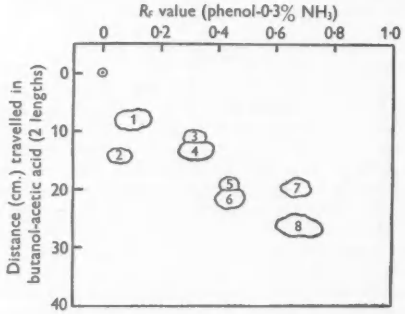

Fig. 5. Chromatogram of fraction $B1\alpha$ (see Table 3). In Figs. 5–16 the fraction was applied in the position marked by the small dotted circle, and the chromatogram run first in the direction represented horizontally, which, except in Fig. 15, was the longer dimension of the filter paper.

Ser.[His, Leu] ($B2\gamma7$) and His.Leu ($B2\gamma8$) were absent, since they had been adsorbed on charcoal and appeared in fraction $B1\gamma$.

The results with fractions $B2\alpha$ and $B2\beta$ (Figs. 10–12; Tables 7 and 8) which are largely confirmatory, are included to show the type of separation that can be obtained with mixtures of about twenty peptides, and also to show that very few peptides were overlooked in the preliminary experiments. The cresol chromatogram of fraction $B2\alpha$ (Fig. 11) is included to demonstrate the positions of the fast-moving spots 21 and 22, which did not appear on the phenol chromatogram.

A large number of solvents was studied for fractionating the basic peptides, most of which move fast on systems such as phenol-0·3% NH_3 and slow on butanol-acetic acid or collidine. Cresol-0·03% NH_3 was eventually found to give good separations (Fig. 13) and is to be recommended for the fractionation of basic peptides and also of larger peptides.

In one experiment fraction $B2\gamma$ was subjected to a further ionophoresis to obtain a fraction containing only arginine peptides. This was carried out in the four-compartment cell (Fig. 2). Compartment 2 contained 1% (w/v) acetic acid and compartment 4 0·1 M-aqueous NH_3. Compartment 3 was maintained at a pink reaction to phenolphthalein. The maximum current was 120 ma. and dropped to 30 ma. The basic fraction (from compartment 4) contained only arginine and Arg.Gly ($B2\gamma5$). The fraction from compartment 3 contained traces of these besides the other basic peptides

Considerable difficulty was experienced in fractionating the fast-moving neutral peptides, which appeared in spots $B2\beta24$ and $B2\beta25$ in Exp. $B2$. The most satisfactory solvents

Table 3. *Peptides from fraction* $B1\alpha$

Spot no. (Fig. 5)	Amino-acids present	Strength of amino-acid after: Hydrolysis	Deamination and hydrolysis	DNP treatment and hydrolysis	Structure
1	$CySO_3H$	× × × ×	?	—	$CySO_3H$.Gly
	Gly	× × × ×	× × × ×	× × ×	
2	Asp	×			[Asp, Glu]
	Glu	×			
3	$CySO_3H$	× ×		×	Val.[$CySO_3H$, Gly]
	Gly	×		×	
	Val	× ×		—	
4	$CySO_3H$	× × ×	× ×	× ×	Val.$CySO_3H$
	Val	× × ×	?	—	
5	$CySO_3H$	×	×	×	Leu.[$CySO_3H$, Gly]
	Gly	×	?	?	
	Leu	×	—	—	
6	$CySO_3H$	× × ×	× × ×	× × ×	Leu.$CySO_3H$
	Leu	× × ×	× ×	—	
7	$CySO_3H$	× ×	×	× ×	Leu.[$CySO_3H$, Gly, Val]
	Gly	× ×	×	× ×	
	Val	× ×	×	× ×	
	Leu	× ×	?	—	
8	$CySO_3H$	× × × ×	× × ×	× × ×	Leu.[$CySO_3H$, Val]
	Val	× × × ×	× × ×	× × ×	
	Leu	× × × ×	×	—	

Spot 1. Yellow colour with ninhydrin which on further heating became grey and then violet.

Spot 8. Eluates of spot 8 from two chromatograms were hydrolysed 4 days at 37° in 11 N-HCl and investigated on a phenol-0·3% NH_3 chromatogram. Besides spots corresponding to cysteic acid, valine, leucine and the unchanged peptide there was a spot having R_F 0·32 which contained valine and cysteic acid and a spot moving faster than leucine which contained valine. Since valine is present in both these dipeptides it must be in the middle of peptide 8, whose structure is therefore Leu.Val.$CySO_3H$.

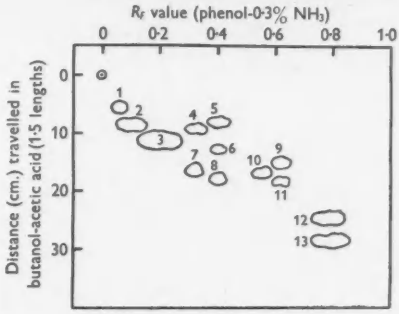

Fig. 6. Chromatogram of fraction $B1\beta$ (see Table 4).

Table 4. *Peptides from fraction* $B1\beta$

Spot no. (Fig. 6)	Amino-acids present	Strength of amino-acid after: Hydrolysis	Deamination and hydrolysis	DNP treatment and hydrolysis	Structure
1	$CySO_3H$	× ×			$CySO_3H$.Gly ($B1\alpha1$)
	Gly	× ×			
2	Asp	× × × ×			Aspartic acid
3	Glu	× × × × ×			Glutamic acid
4	$CySO_3H$	×			Val.[$CySO_3H$, Gly] ($B1\alpha3$)
	Gly	× ×			
	Val	×			
5	$CySO_3H$	×			[$CySO_3H$, Glu, His, Leu]
	Glu	×			
	His	×			
	Leu	×			
6	$CySO_3H$	×			Leu.[$CySO_3H$, Gly] ($B1\alpha5$)
	Gly	×			
	Leu	×			
7	Glu	×			Glu.Ala ($B1\delta4$)
	Ala	×			
8	Asp	×	?	×	Val.Asp
	Val	×	—	—	
9	$CySO_3H$	×			Leu.[$CySO_3H$, Gly, Val] ($B1\alpha7$)
	Gly	×			
	Val	×			
	Leu	×			
10	Asp	× ×	×	× ×	Val.[Asp, Glu]
	Glu	× ×	×	× ×	
	Val	× ×	—	—	
11	$CySO_3H$	×			Leu.[$CySO_3H$, Val] ($B1\alpha8$)
	Val	×			
	Leu	×			
12	Asp	× × × ×		× × ×	Phe.[Asp, Glu, Val]
	Glu	× × × ×		× × × ×	
	Val	× × × ×		× × × ×	
	Phe	× × × ×		×	
13	Asp	× × × ×		× ×	Phe.[Asp, Val]
	Val	× × × ×		× ×	
	Phe	× × × ×		—	

Table 5. *Peptides from fraction B 1γ*

Spot no. (Figs. 7 and 8)	Amino-acids present	Strength of amino-acid after: Hydrolysis	Deamination and hydrolysis	DNP treatment and hydrolysis	Structure
1	Glu	× ×		?	Glu.His
	His	× ×		×*	
2	Glu	× ×			[Glu, Gly, Arg]
	Gly	× ×			
	Arg	× ×			
3	Glu	× × ×	×		Glu.Arg + spot 2
	Gly	×	?		
	Arg	× × ×	× × ×†		
4	$CySO_3H$	× × ×	× ×	× ×	His.[$CySO_3H$, Leu]
	His	× × ×	?	—	
	Leu	× × ×	× × ×	× ×	
5	Thr	× × × ×			Thr.[Ala, Lys, Pro] (*B*2γ6)
	Ala	× × × ×			
	Lys	× × × ×			
	Pro	× × ×			
6	Asp	×		×	Phe.[Asp, Glu, His, Val]
	Glu	×		×	
	His	×		‡	
	Val	×		×	
	Phe	×		—	
7	Glu	× ×			[Glu, His, Leu]
	His	× ×			
	Leu	× ×			
8	Leu	× ×			His.Leu (*B*2γ8)
	His	× ×			
9	Glu	×			Val.Glu (*B*1δ7)
	Val	×			
10	Glu	×	×		Val.[Ala, Glu]
	Ala	×	×		
	Val	×	—		
11	Tyr	×			Tyrosine
12*a*	Gly	×			Gly.Phe
	Phe	×			
12*b*	Asp	× ×		×	See below
	Glu	× ×		×	
	Gly	× ×		?	
	Val	× ×		×	
	Phe	× × ×		× ×	
13	Leu	×			Leucine + phenylalanine
	Phe	× ×			
14	Glu	× × ×			Leu.[Glu, Val] (*B*2β14)
	Val	× × ×			
	Leu	× × ×			
15	Glu	×			Leu.[Glu, Ala, Val] (*B*2β15)
	Ala	×			
	Val	×			
	Leu	×			
16	Ala	× ×			Ala.Leu (*B*1δ11)
	Leu	× ×			
17	Unidentified mixture of fast-moving peptides, containing Phe, Leu, Val, Tyr, Ala				

* DNP derivative.
† Arginine decomposition product (R_F, 0·73 with phenol).
‡ *im*-DNP-Histidine not distinguished on this phenol chromatogram.

Spots 2 and 3 appear to be mixtures of a tripeptide [Glu, Gly, Arg] and a dipeptide [Glu, Arg], the latter moving slightly faster on butanol-acetic acid.

Spot 7, obtained from phenol chromatograms, sometimes contains serine probably due to the presence of Ser.[His, Leu] (*B*2γ7).

Spot 8 sometimes contains glutamic acid from spot 7.

Spot 12*a* gives a yellow colour with ninhydrin, suggesting glycine is terminal. The DNP treatment was done only on the mixture in spot 12*b*, which clearly demonstrates that glycine must be terminal.

Spot 12*b* is a mixture of 12*a* with another peptide, probably Phe.[Asp, Glu, Val] (*B*1β12). This latter did not appear on the cresol chromatogram (Fig. 8).

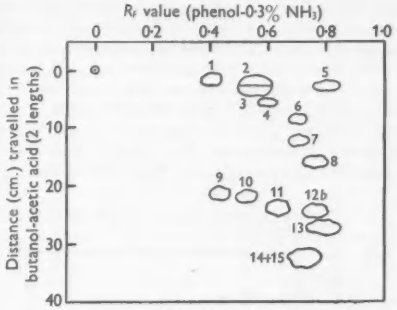
R_F value (phenol-0·3% NH_3)
0 0·2 0·4 0·6 0·8 1·0
Distance (cm.) travelled in butanol-acetic acid (2 lengths)
0 10 20 30 40
1 2 3 4 5 6 7 8 9 10 11 12b 13 14+15

Fig. 7. Chromatogram of fraction $B1\gamma$ using phenol-0·3% NH_3 (see Table 5).

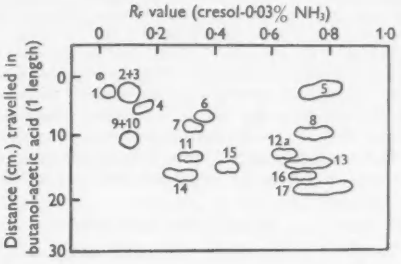
R_F value (cresol-0·03% NH_3)
0 0·2 0·4 0·6 0·8 1·0
Distance (cm.) travelled in butanol-acetic acid (1 length)
0 10 20 30
2+3 1 4 9+10 6 7 11 15 14 5 8 12a 13 16 17

Fig. 8. Chromatogram of fraction $B1\gamma$ using cresol-0·03% NH_3 (see Table 5).

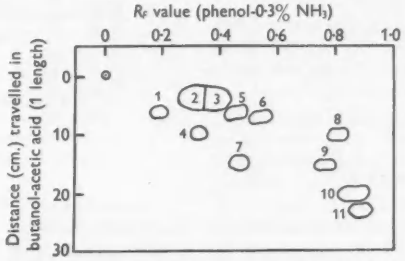

Fig. 9. Chromatogram of fraction $B1\delta$ (see Table 6).

Table 6. *Peptides from fraction B* 1δ

Spot no. (Fig. 9)	Amino-acids present	Strength of amino-acid after: Hydrolysis	Deamination and hydrolysis	DNP treatment and hydrolysis	Structure
1	Glu	×	×	×	Gly.Glu
	Gly	×	—	?	
2	Ser	× × ×			Serine
	Gly	×			
3	Gly	× × × ×			Glycine
4	Glu	×	?		Glu.Ala
	Ala	×	×		
5	Thr	×			Threonine
6	Ala	× × × ×			Alanine
7	Glu	× ×	× ×	× ×	Val.Glu
	Val	× ×	—	?	
8	Thr	× ×	?	—	Thr.Pro
	Pro	× ×	× ×	× × ×	
9	Val	× ×			Valine
10	Leu	× × × ×			Leucine
11	Leu	× × × ×	× × × ×	× × × ×	Ala.Leu
	Ala	× × × ×	× ×	×	

Spot 1 gives a yellow colour with ninhydrin.

Spot 7 gives a very weak reaction with ninhydrin and a relatively strong fluorescence.

Spot 8 gives a strong yellow colour with ninhydrin.

Spot 10 is often contaminated with traces of serine, which is located in the slower moving parts of the spot. If *m*-cresol is used instead of phenol the leucine spot is pure and the serine can be found together with leucine in a slower moving faint spot (R_F, 0·57). This is probably Ser.Leu derived from fraction *A*, from which it can be obtained in large amounts.

Spot 11 is often contaminated with threonine, which we have not yet been able to account for satisfactorily.

were collidine *tert.*-amyl alcohol (2-methylbutan-2-ol) and benzyl alcohol, but there seemed to be too many of these closely related peptides to give clear results. The best results were obtained with fraction $B3\beta$ (Fig. 14, Table 10), from which some of the more strongly adsorbed peptides had been removed by charcoal treatment. The very large number of these peptides containing tyrosine, valine, leucine and phenylalanine is partly due to the partial breakdown of tyrosine to TyrX, which means that two spots are obtained for each tyrosine peptide. It also suggests that these non-polar residues are grouped together in the fraction *B* chains.

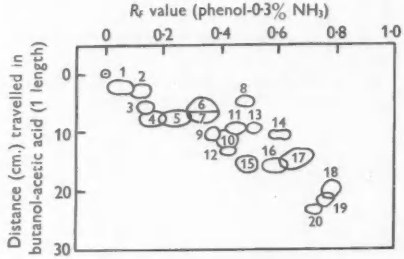

Fig. 10. Chromatogram of fraction $B2\alpha$ using phenol-0·3% NH_3 (see Table 7).

The results with the other fractions of Exp. $B3$ are not recorded, since they were essentially the same as the $B2$ fractions. Fraction $B3\delta$, which was adsorbed on charcoal, contained mainly [$CySO_3H$, Tyr, Val, Leu] ($B2\alpha21$) and Phe.[Asp, Glu, Val] ($B1\beta12$) and fast-moving peptides containing tyrosine, valine, leucine and phenylalanine.

The peptides found in fraction $B4\alpha$ are listed in Table 11. Fractions $B4\beta$ and $B4\gamma$ yielded a few new peptides, which are recorded in Table 12. Other peptides are not recorded. Spot $B4\beta1$ was obtained from a cresol-0·03% NH_3/butanol-acetic acid chromatogram and ran at the same rate in both solvents as His.[$CySO_3H$, Leu] (Peptide $B2\beta6$, Fig. 12). Peptide $B4\beta2$ was a fast-moving spot on a collidine, *tert.*-amyl alcohol chromatogram. The peptides from $B4\gamma$ were obtained from the chromatogram illustrated in Fig. 15.

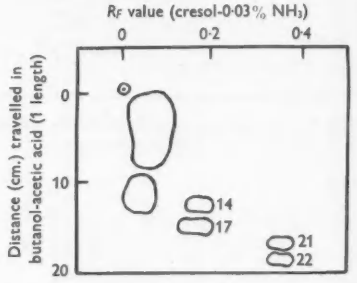

Fig. 11. Chromatogram of fraction $B2\alpha$ using cresol-0·03% NH_3 (see Table 7).

Fraction $B5\alpha$ contained very little material and was not investigated in detail. $B5\beta$ contained free glycine and alanine (but no other free amino-acids), Gly.[Glu, Orn] ($B5\gamma1$), Gly.Glu ($B1\delta1$) and a large amount of fast-moving peptides. There was also a yellow spot ($R_F = 0{\cdot}55$ in cresol-0·03% NH_3 and a little faster than alanine on butanol-acetic acid) which gave glycine and proline on hydrolysis. It

Table 7. *Peptides from fraction* $B2\alpha$

Spot no. (Figs. 10 and 11)	Strength	Structure	Identical with
1	× × × ×	Cysteic acid	—
2	× × × ×	$CySO_3H$.Gly	$B1\alpha1$
3	× ×	Aspartic acid	—
4	× ×	Gly.Glu	$B1\delta1$
5	× × ×	Glutamic acid	—
6	× ×	Val.[$CySO_3H$, Gly]	$B1\alpha3$
7	×	Val.$CySO_3H$	$B1\alpha4$
8	×	[$CySO_3H$, Glu, His, Leu]	$B1\beta5$
9	× ×	Glu.Ala	$B1\delta4$
10	× × ×	Leu.$CySO_3H$	$B1\alpha6$
11	× × ×	Leu.[$CySO_3H$, Gly]	$B1\alpha5$
12	× ×	Val.Asp	$B1\beta8$
13	×	Alanine	—
14	× × ×	Leu.[$CySO_3H$, Gly, Val]	$B1\alpha7$
15	× × × ×	Val.Glu	$B1\delta7$
16	×	Val.[Ala, Glu]	$B1\gamma10$
17	× × × ×	Leu.[$CySO_3H$, Val]	$B1\alpha8$
18	× ×	Phe.[Asp, Glu, Val]	$B1\beta12$
19	×	Leu.[Glu, Ala, Val]	$B2\beta15$
20	× ×	Leu.[Glu, Val]	$B2\beta14$
21	×	[$CySO_3H$, TyrX, Val, Leu]	—
22	×	[$CySO_3H$, TyrXX, Val, Leu]	—

Spot 8 sometimes contains a trace of glycine.

Spot 22. Besides cysteic acid, valine and leucine, a new spot was present that had R_F, 0·53 (phenol-0·3% NH_3) and moved as fast as leucine on butanol-acetic acid. It was not detected in any other peptides and is assumed to be another breakdown product of tyrosine (TyrXX).

is assumed that this was formed by the action of the alkali on Thr.Pro (*B*188). Wieland & Wirth (1949) have shown that glycine is formed from threonine by the action of alkali.

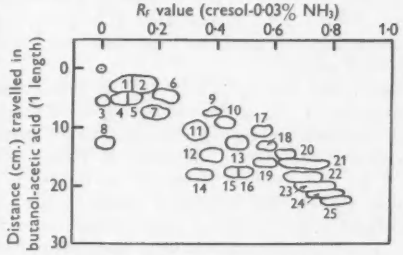

Fig. 12. Chromatogram of fraction *B*2β (see Table 8).

The results with fraction *B*5γ are recorded in Fig. 16 and Table 13. Besides the peptides shown there were a few unidentified spots that moved faster on butanol-acetic acid. The amino-acid referred to as Orn moved at the same R_F values as ornithine in phenol and butanol-acetic acid and is assumed to be a breakdown product of arginine.

DISCUSSION

Methods of hydrolysis

The present results support the conclusion that concentrated hydrochloric acid at low temperatures is the most generally useful method of partial hydrolysis (Synge, 1943). No apparent destruction of amino-acids occurs, and a wide variety of lower peptides is produced. The disadvantage of using only one method is that it is difficult to obtain peptides which contain the most labile bonds, and which are required to work out a complete sequence of amino-acids. The bonds that are most labile under these conditions are those involving the amino groups of serine and threonine residues (Desnuelle & Casal, 1948) and no peptides have been found containing these bonds intact. Bonds involving glycine residues are also somewhat labile, but some such peptides were obtained. Other methods of hydrolysis were therefore studied in the hope of supplementing the results from the hydrolysates made with concentrated hydrochloric acid and also in the hope of obtaining a rather less complex mixture of the fast-moving neutral peptides, suitable for fractionation. While neither of these objectives was attained, a few new peptides were detected.

In studying the terminal peptides (Sanger, 1949*b*) it was found that glycine peptides were relatively more stable to dilute acid than to concentrated acid. This is also apparent from the present work. If fraction *B*4α (Table 11) is compared with fraction *B*1α (Table 3) it will be seen that the dilute-acid hydrolysate contains a higher proportion of the peptides in which the $CySO_3H$.Gly bond is intact. Similarly, [$CySO_3H$, Gly, His, Leu] (*B*4β1) occurs in

Table 8. *Peptides from fraction B*2β

Spot no. (Fig. 12)	Strength	Structure	Identical with
1	× ×	Glu.His	*B*1γ1
2	× × × ×	Glu.Arg + [Glu, Gly, Arg]	*B*1γ2 + *B*1γ3
3	×	Gly.Glu	*B*1δ1
4	× × × ×	Serine + glycine	—
5	× × ×	Glycine + threonine	—
6	× ×	His.[$CySO_3H$, Leu]	*B*1γ4
7	× × × ×	Alanine	—
8	×	Val.Glu + Val.[Ala, Glu]	*B*1γ9 + *B*1γ10
9	×	Phe.[Asp, Glu, His, Val]	*B*1γ6
10	× ×	[Glu, Ser, His, Val, Leu]	*B*4γ3
11	× ×	Tyrosine	—
12	×	TyrX	—
13	× ×	Valine	—
14	× × × ×	Leu.[Glu, Val]	—
15	× ×	Leu.[Glu, Ala, Val]	—
16	× ×	Phe.[Asp, Val]	*B*1β13
17	× × ×	Thr.Pro	*B*1δ8
18	× ×	[Glu, His(?)Val, Leu]	—
19	× ×	Phe.[Asp, Glu, Val]	*B*1β12
20	× ×	Gly.Phe	*B*1γ12*a*
21	× × × ×	Phenylalanine + leucine	—
22	× × × ×	Ala.Leu	*B*1δ11
23	× × ×	Ala.[Tyr, Leu]	*B*3β9

Spot 6 contains a trace of glycine, probably due to [His, Leu, $CySO_3H$, Gly] (*B*4β1).

Spot 10. Glutamic acid, histidine and leucine are slightly stronger so probably contains peptide *B*1γ7 ([Glu, His, Leu]).

Spot 14. After DNP treatment only valine and glutamic acid were present.

Spot 15. *N*-terminal residue determined on mixture in *B*3β4.

Spot 18. The histidine was not clearly distinguished on this phenol chromatogram as it comes very close to the valine spot, but it is probably significant since the peptide cannot be [Glu, Val, Leu] which is in spot 14.

Spots 24, 25. Unidentified mixtures of peptides containing tyrosine, valine, leucine and phenylalanine.

the dilute-acid hydrolysate, whereas the corresponding peptide without glycine ($B2\beta6$) is obtained with concentrated hydrochloric acid hydrolysis. The presence of several new histidine peptides in fraction

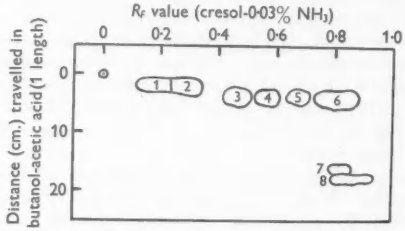

Fig. 13. Chromatogram of fraction $B2\gamma$ (see Table 9).

$B4\gamma$ (Table 12) suggests that bonds involving histidine may also be relatively more stable to dilute acid. The bonds involving the amino groups of serine and threonine, however, appear to be labile to dilute acid also, and no peptides were obtained in which they were intact. The large amount of free aspartic acid ($B4\alpha2$), compared with only traces of free cysteic acid (possibly present in spot $B4\alpha1$), is interesting in view of the results of Partridge & Davis (1950), who demonstrated a preferential release of aspartic acid when proteins were boiled with weak acids.

The hydrolysis in alkali led to considerable destruction of the amino-acids, apparently while they were still in peptide linkage, which greatly complicates the results. Wieland & Wirth (1949) found no destruction of serine or threonine on heating 12 hr. with saturated barium hydroxide, conditions which are more violent than those used here. However, the residues seem considerably more labile when in peptide linkage. No free serine or threonine was detected, and the Gly.Pro was almost certainly derived from Thr.Pro. Arginine appears to be converted almost quantitatively to ornithine since no free arginine was detected, whereas free ornithine and a peptide containing ornithine ($B5\gamma1$) were present.

Table 9. *Peptides from fraction $B2\gamma$*

Spot no. (Fig. 13)	Amino-acids present	Strength of amino-acid after: Hydrolysis	Deamination and hydrolysis	DNP treatment and hydrolysis	Structure
1	Ser	× × ×			2+lysine
	Lys	×			
	His	× × ×			
2	Ser	× × × ×	×	—	Ser.His
	His	× × × ×	× × ×	× ×*	
3	His	× ×			Arginine+histidine
	Arg	× × ×			
4	Ala	× × ×		× ×	Lys.Ala
	Lys	× × ×		—	
5	Gly	× × ×		× ×	Arg.Gly
	Arg	× × ×		—	
6	Thr	× × × × ×		×	Thr.[Ala, Lys, Pro]
	Ala	× × × × ×		× × ×	
	Lys	× × × × ×		× × ×*	
	Pro	× × × × ×		× × ×	
7	Ser	× ×		—	Ser.[His, Leu]
	His	× ×		×*	
	Leu	× ×		×	
8	His	× × ×	?	—	His.Leu
	Leu	× × ×	× ×	× ×	

* DNP derivative.

This fraction also contained traces of free neutral amino-acids, which we have always found in the basic fractions from the ionophoresis. These are not recorded.

Spot 1. Part of the Ser.His spot contains free lysine. These two are well separated if 0·3% NH_3 is used with the cresol, when the lysine travels much faster. However, the other peptides are badly separated and only Ser.His appears as a single pure spot.

Spot 4. Hydrolysis of the DNP derivative gave bis-DNP-lysine.

Spot 5. The DNP derivative of this basic peptide could be extracted (though not rapidly) into ethyl acetate from acid solution, in contrast to DNP-arginine. It was not well adsorbed on talc from acid solution, but could be adsorbed better from neutral solution.

Spot 6. Contains traces of glycine which has probably arisen by partial breakdown of the *N*-terminal threonine.

Methods of group separation

Probably none of the experiments described represent the most satisfactory procedure for fractionating the mixture concerned. Exp. *B* 1, in which most of the aromatic peptides were lost by adsorption on the resin, was designed primarily to separate cysteic acid peptides and it was only later decided to study the other fractions.

The four-compartment cell of Synge was found to give very satisfactory separations into acidic, basic and neutral peptides with only a little overlapping. Under the conditions described many of the simple peptides of glutamic acid were found in the neutral fraction (*B* 2β) as well as in the acidic fraction (*B* 2α). By maintaining the specimen compartment at a slightly higher pH this overlapping could be avoided and all the glutamic acid peptides were then found in the acidic fraction, but under these conditions the neutral fraction contained histidine peptides.

The ion-exchange resin gave a good separation of peptides of cysteic acid, which are less strongly adsorbed than free cysteic acid. No clear separation of peptides containing aromatic residues was obtained by adsorption on charcoal. Several other peptides were also strongly adsorbed. Most of these

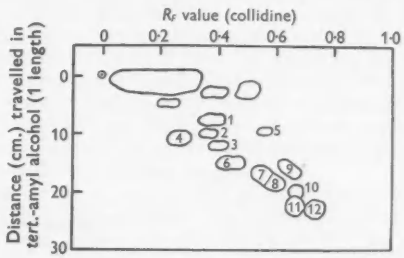

Fig. 14. Chromatogram of fraction *B* 3β (see Table 10).

Table 10. *Peptides from fraction B* 3β

Spot no. (Fig. 14)	Amino-acids present	Strength of amino-acid after Hydrolysis	Strength of amino-acid after DNP treatment and hydrolysis	Structure
1	Asp	× ×	×	Phe.[Asp, Glu, Val] (*B* 1β12) + Gly.Phe (*B* 1γ12*a*)
	Glu	× ×	×	
	Gly	× ×	?	
	Val	× ×	×	
	Phe	× × ×	×	
2	Asp	× ×		Phe.[Asp, Val] (*B* 1β13)
	Val	× ×		
	Phe	× ×		
3	Ala	× × × × ×		Ala.Leu (*B* 1δ11)
	Leu	× × × × ×		
4	Glu	× × × ×	× × ×	Leu.[Glu, Val] (*B* 2β14) + Leu.[Glu, Ala, Val] (*B* 2β15)
	Ala	× ×	×	
	Val	× × × ×	× × ×	
	Leu	× × × ×	?	
5	TyrX	× ×		TyrX
6	Phe	× × ×		Phenylalanine + leucine
	Leu	× × × ×		
7	Val	× × ×	× × ×	Leu.Val
	Leu	× × ×	×	
8	Val	× ×		[Val, Phe] (*B* 4β2) + 7
	Leu	× ×		
	Phe	×		
9	Ala	× ×	—	Ala.[Tyr, Leu]
	Tyr	× ×	?*	
	Leu	× × ×	×	
10	TyrX + Tyr	× ×	?	[TyrX, Leu] [Tyr, Leu]
	Leu	× ×	×	
11	Tyr	?		Mixture
	Val	×		
	Leu	× ×		
	Phe	× ×		
12	Tyr	×		[Tyr, Val, Leu]
	Val	× ×		
	Leu	× ×		

* DNP derivative.

Table 11. *Peptides from fraction B 4α*

Peptide	Strength	Structure	Identical with
1	× × ×	$CySO_3H$.Gly	*B* 1α1
2	× × × ×	Aspartic acid	—
3	× × ×	Glutamic acid	—
4	× ×	Val.[$CySO_3H$, Gly]	*B* 1α3
5	× ×	Leu.[$CySO_3H$, Gly]	*B* 1α5
6	×	Leu.$CySO_3H$	*B* 1α6
7	× ×	Leu.[$CySO_3H$, Gly, Val]	*B* 1α7
8	×	Leu.[Val, $CySO_3H$]	*B* 1α8

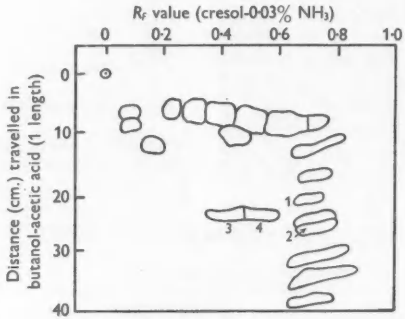

Fig. 15. Chromatogram of fraction *B* 4γ. The chromatogram was run first down the short direction of the filter paper with cresol-0·03% NH_3 and then down the long direction with butanol-acetic acid (see Table 12).

Table 12. *Peptides from fractions B 4β and B 4γ*

Peptide	Amino-acids present	Strength of amino-acid after: Hydrolysis	Strength of amino-acid after: DNP treatment and hydrolysis	Structure
β1	$CySO_3H$	× × ×		[$CySO_3H$, Gly, His, Leu]
	Gly	× × ×		
	His	× × ×		
	Leu	× × ×		
β2	Val	× × ×		[Val, Phe]
	Phe	× ×		
γ1*	Ser	× ×		Ser.[His, Leu] (*B* 2γ7)
	His	× ×		
	Leu	× × ×		
γ2*	Ser	×	?	Ser.[His, Val, Leu]
	His	× ×	†	
	Val	× ×	× ×	
	Leu	× ×	× ×	
γ3*	Glu	× ×		[Glu, Ser, His, Val, Leu]
	Ser	× ×		
	His	× ×		
	Val	× × ×		
	Leu	× × ×		
γ4*	Glu	× ×	×	Ser.[Glu, Ala, His, Val, Leu]
	Ser	×	?	
	Ala	× ×	×	
	His	× ×	†	
	Val	× ×	×	
	Leu	× ×	×	

* Spot nos. as in Fig. 15. These gave yellow colour with ninhydrin.
† Run on a phenol chromatogram, so *im*-DNP-His not observed.

contained both an acidic and a basic residue. Thus [Glu.Arg] ($B1\gamma3$) and Glu.His ($B1\gamma1$) were adsorbed, whereas Arg.Gly ($B2\gamma5$) and Ser.[His, Leu] ($B2\gamma7$) were not. Similarly, it was found that the DNP derivative of Glu.Arg could be adsorbed from acid solution on to talc, whereas the derivative of Arg.Gly was only weakly adsorbed, in contrast to the behaviour of most DNP peptides.

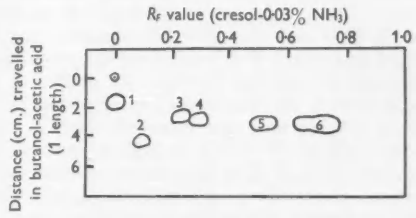

Fig. 16. Chromatogram of fraction $B5\gamma$ (see Table 13).

Probably the best method of carrying out the preliminary group separations on these hydrolysates would be to obtain first the acidic, neutral and basic fractions by ionophoresis. The acidic one could be further fractionated first with charcoal and then on the ion-exchange resin to separate peptides of cysteic acid. The neutral one could be separated into two fractions by charcoal adsorption, and the basic one could be subjected to a second ionophoresis at a higher pH to separate arginine peptides.

From the paper chromatograms it may be concluded that in general mixtures containing up to about twenty peptides (fractions $B2\alpha$ and $B2\beta$) may be satisfactorily fractionated, though for preliminary work it is preferable to have fewer present.

Structure of the peptides

The most probable composition of fraction B is given in Table 14. This is obtained from a consideration of the composition of insulin (Tristram, 1949) and of the very approximate analysis of fraction A (Sanger, 1949*a*), and may therefore not be very accurate. The value for cysteic acid was determined directly on fraction B by Rees by determination of the S content, and the figures for histidine, arginine, lysine and threonine are reliable since fraction A contains none of these residues.

N-Terminal peptide. The results with the DNP peptides demonstrated the presence of the *N*-terminal sequence, Phe.Val.Asp.Glu (Sanger, 1949*b*). The present results offer ample confirmation for this sequence, which may be deduced as follows:

Table 14 indicates that fraction B contains only one aspartic acid residue, so that all peptides con-

Table 13. *Peptides from fraction B 5γ*

Spot no. (Fig. 16)	Amino-acids present	Strength of amino-acid after: Hydrolysis	Strength of amino-acid after: DNP treatment and hydrolysis	Structure
1	Glu	× × ×	× × ×	Gly.[Glu, Orn]
	Gly	× × ×	×	
	Orn	× × × ×	× × ×*	
2	Gly	×		Glycine
3	Orn	×		Ornithine
4	Lys	×		Lysine
5	Ala	× ×		Lys.Ala ($B2\gamma4$)
	Lys	× ×		
6	Ala	× × ×	× × ×	Pro.[Ala, Lys]
	Lys	× × ×	× ×*	
	Pro	× × ×	?	

* DNP derivative.

Spot 1. DNP derivative hydrolysed 6 hr. This peptide was also found in the neutral fraction ($B5\beta$), which is where one would expect to find it.

Spot 6. DNP derivative hydrolysed 12 hr. with 11N-HCl at 105°.

Table 14. *Probable amino-acid composition of fraction B*

Amino-acid	No. of residues/mol.
Cysteic acid	2
Aspartic acid	1
Glutamic acid	3–4
Serine	1
Glycine	3
Threonine	1
Alanine	2
Tyrosine	2
Valine	2
Leucine	3–4
Phenylalanine	3
Proline	1
Histidine	2
Lysine	1
Arginine	1

taining aspartic acid should fit into a single sequence. Six such peptides have been found: [Asp, Glu] ($B\,1\alpha2$), Val.Asp ($B\,1\beta8$), Val.[Asp, Glu] ($B\,1\beta10$), Phe.[Val, Asp, Glu] ($B\,1\beta12$), Phe.[Val, Asp] ($B\,1\beta13$) and Phe.[Asp, Glu, His, Val] ($B\,1\gamma6$). It can be seen that the structure of the first five peptides can only be explained on the basis of the above sequence. Phe.Val ($B\,4\beta2$) is also derived from this sequence.

Peptide $B\,1\gamma6$ moves at different rates on the chromatograms from the other peptides of aspartic acid so must contain histidine as an integral part and not as an impurity. It can also be seen that none of the other histidine peptides would be in this position. Its structure must therefore be Phe.Val.Asp.Glu.His. This is also supported by the identification of Glu.His ($B\,1\gamma1$). The fact that no peptides of aspartic acid have been found that do not fit this sequence confirms that fraction *B* contains only one aspartic acid residue.

Peptides of lysine. With the DNP method it was shown that the lysine was present in the sequence Thr.Pro.Lys.Ala, and this sequence is also confirmed in the present work by the identification of the following peptides: Thr.Pro ($B\,1\delta8$), Lys.Ala ($B\,2\gamma4$), Thr.[Pro, Lys, Ala] ($B\,2\gamma6$), Pro.[Lys, Ala] ($B\,5\gamma6$). Since fraction *B* only contains one lysine and one threonine residue this establishes the sequence. No other peptides containing proline have been found, indicating that there is also only one proline residue. It appears that the Thr.Pro bond is unusually resistant to concentrated hydrochloric acid since large amounts of Thr.Pro and the tetrapeptide were found, whereas only traces of free proline could be detected. The tripeptide Pro.Lys.Ala was not found in the concentrated acid hydrolysate, whereas it was in the alkaline hydrolysate.

Peptides of arginine. Two dipeptides of arginine were detected: Glu.Arg ($B\,1\gamma3$) and Arg.Gly ($B\,2\gamma5$). Since there is only one arginine residue this establishes the sequence Glu.Arg.Gly. In peptide $B\,5\gamma1$ (Gly.[Glu, Orn]) the ornithine is evidently derived from arginine. Since glycine is the *N*-terminal residue its structure must be Gly.Glu.Orn, and the order in fraction *B* is therefore Gly.Glu.Arg.Gly. Gly.Glu ($B\,1\delta1$) was detected also and appears to originate from this sequence. This structure could give rise to two tripeptides containing glycine, glutamic acid and arginine. Peptide $B\,1\gamma2$ is either one or both of these. Difficulties in deaminating this peptide may have been due to its being a mixture.

Peptides of cysteic acid. Three dipeptides containing cysteic acid have been detected: $CySO_3H$.Gly ($B\,1\alpha1$), Val.$CySO_3H$ ($B\,1\alpha4$) and Leu.$CySO_3H$ ($B\,1\alpha6$). Since fraction *B* contains only two cysteic acid residues, all peptides in which cysteic acid is not *N*-terminal must therefore contain the sequences Val.$CySO_3H$ or Leu.$CySO_3H$. Peptide $B\,1\alpha3$ is therefore Val.$CySO_3H$.Gly and peptide $B\,1\alpha5$ is Leu.$CySO_3H$.Gly. The only possible structure for $B\,1\alpha8$, which contains valine but no glycine is Leu.Val.$CySO_3H$ and this was also demonstrated by partial hydrolysis. This establishes the presence of the sequence Leu.Val.$CySO_3H$.Gly in fraction *B*, and peptide $B\,1\alpha7$ clearly has this structure. Leu.Val ($B\,3\beta7$) has also been detected. Peptide $B\,1\gamma4$ which contains no valine or glycine can only be His.Leu.$CySO_3H$. This establishes a second tetrapeptide sequence His.Leu.$CySO_3H$.Gly, and peptide $B\,4\beta1$ presumably has this structure. The presence of His.Leu ($B\,2\gamma8$) is further evidence for this sequence. Another peptide containing cysteic acid is $B\,1\beta5$ ([$CySO_3H$, Glu, His, Leu]) and the question arises as to whether it is a pure peptide or could be a mixture. The cysteic acid cannot be present as the free amino-acid, which moves slower on phenol; therefore it must be in a peptide. No valine or glycine is present so that it must be present in the sequence Leu.$CySO_3H$. The dipeptide Leu.$CySO_3H$ goes considerably faster on butanol-acetic acid and would appear below spot 6 (Fig. 6), so that the cysteic acid in spot 5 must be present in the form His.Leu.$CySO_3H$. This tripeptide ($B\,1\gamma4$) goes faster on phenol (Fig. 7) so that the cysteic acid must be present in the tetrapeptide Glu.His.Leu.$CySO_3H$. In support of this structure we have also found the peptides Glu.His ($B\,1\gamma1$), and [Glu, His, Leu] ($B\,1\gamma7$). The sequence in fraction *B* is therefore Glu.His.Leu.$CySO_3H$.Gly. The fact that spot $B\,2\alpha8$ ([$CySO_3H$, Glu, His, Leu]) sometimes contains a trace of glycine suggests that the pentapeptide may also be present.

Peptide $B\,2\alpha21$ ([$CySO_3H$, Tyr, Val, Leu]) moves considerably faster on cresol than any of the other cysteic acid peptides including Leu.Val.$CySO_3H$ ($B\,2\alpha17$) (Fig. 11). The tyrosine must therefore be present as part of the peptide, and since it contains no glycine or histidine the cysteic acid must be in the sequence Leu.Val.$CySO_3H$ and must be the *C*-terminal residue. The structure of the peptide is thus Tyr.Leu.Val.$CySO_3H$, and the order in fraction *B* must be Tyr.Leu.Val.$CySO_3H$.Gly. This sequence would be expected to give rise to Tyr.Leu and Tyr.Leu.Val. Spots $B\,3\beta10$ ([Tyr, Leu]) and $B\,3\beta12$ ([Tyr, Val, Leu]) probably contain these peptides, though it is not certain that they are not mixtures.

Peptides of valine and alanine. According to Table 14 fraction *B* should only contain two valine residues. One of these is present in the *N*-terminal sequence Phe.Val.Asp.Glu.His, another in the sequence Tyr.Leu.Val.$CySO_3H$.Gly. However there are other peptides containing valine which do not fit these sequences. It is therefore necessary to assume either that fraction *B* contains three valine

Table 15. *Peptides obtained from phenylalanyl chain of insulin (fraction B)*

| | | |

residues or else that it is not homogeneous. The former explanation is preferred.

Besides the sequences already considered, valine is present in the following peptides: Val.Glu ($B1\delta7$), Val.[Ala, Glu] ($B1\gamma10$), Leu.[Glu, Val] ($B2\beta14$) and Leu.[Val, Glu, Ala] ($B2\beta15$). The only sequence fitting all these peptides is Leu.Val.Glu.Ala. This sequence may also be deduced from a consideration of the peptides of alanine. Fraction *B* contains two alanine residues; one is at the end of the sequence Thr.Pro.Lys.Ala. Other peptides are Ala.Leu ($B1\delta11$), Glu.Ala ($B1\delta4$), Val.[Ala, Glu] ($B1\gamma10$), Leu.[Val, Glu, Ala] ($B2\beta15$) and Ala.[Tyr, Leu] ($B3\beta9$). Any peptide containing alanine, which is not *N*-terminal, must either contain Lys.Ala or Glu.Ala. $B1\gamma10$ is therefore Val.Glu.Ala and $B2\beta15$ is Leu.Val.Glu.Ala. The position of Ala.Leu or of Ala.[Tyr, Leu] cannot be deduced and they need not necessarily be from the same sequence.

Peptides of histidine. Two sequences containing histidine have already been considered: Phe.Val.Asp.Glu.His and Glu.His.Leu.$CySO_3H$.Gly. Besides Glu.His and His.Leu, the only other dipeptide of histidine is Ser.His ($B2\gamma2$). Since there are only two histidine residues and one serine residue in fraction *B*, the tripeptide $B2\gamma7$ (Ser.[His, Leu]) can only be Ser.His.Leu. The above two pentapeptide sequences, which both contain Glu.His but not Ser.His must therefore be parts of the same sequence, so that the actual order in fraction *B* is Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly. Further evidence for this sequence was obtained by an extension of the DNP technique. A dilute-acid hydrolysate of the DNP derivative of fraction *B* was fractionated on a buffered Hyflo column using ethyl acetate. Two slow-moving bands were present; one contained DNP-phenylalanine, valine, aspartic acid, glutamic acid, *im*-DNP-histidine and leucine and the second contained these six residues together with cysteic acid and glycine. This in itself could not be considered as direct evidence as they could have been mixtures of terminal DNP-phenylalanyl peptides with peptides of *im*-DNP-histidine, but it adds support to the above conclusion. The establishment of this sequence depends chiefly on the two large peptides $B1\gamma6$ (Phe.[Val, Asp, Glu, His]) and $B1\beta5$ ([Glu, His, Leu, $CySO_3H$]) which are present in rather small amounts and therefore cannot be regarded as completely proved. Ample confirmation was, however, obtained from the results with enzymic hydrolysates (Sanger & Tuppy, 1951).

Further peptides containing histidine were present in the dilute-acid hydrolysate (fraction $B4\gamma$). Peptide $B4\gamma2$ (Ser.[His, Val, Leu]) must contain the sequence Ser.His.Leu and is therefore Ser.His.Leu.Val. The four peptides of fraction $B4\gamma$, shown in Fig. 15, gave a yellow-grey colour with ninhydrin which is characteristic of peptides containing serine as the *N*-terminal residue, and the DNP results showed this to be the case for $B4\gamma4$. Thus the structure of $B4\gamma3$ must be Ser.His.Leu.Val.Glu and of $B4\gamma4$ Ser.His.Leu.Val.Glu.Ala. This sequence may also be derived by considering the valine peptides. The three valine residues are present as Phe.Val.Asp, Tyr.Leu.Val.$CySO_3H$, Leu.Val.Glu.Ala. The latter is the only one to which Ser.His.Leu.Val could be combined, so that the order in fraction *B* must be Ser.His.Leu.Val.Glu.Ala. Spot $B2\beta18$ ([Glu, His, Val, Leu]) is probably His.Leu.Val.Glu derived from this sequence.

Other peptides. In Table 15 are listed all the peptides detected in this work and the sequences which have been deduced in the foregoing discussion. We have included all peptides found in the hydrolysates with the exception of some of the fast-moving peptides such as are present in spots $B2\beta24$ and $B2\beta25$, which could not be identified. Apart from these sequences there are only three peptides which are not included in the five sequences listed. These are Ala.Leu, Gly.Phe, and Ala.[Tyr, Leu].

According to Table 14 all the amino-acids of fraction *B* can be accounted for in the above sequences except for one tyrosine, two phenylalanine and possibly one glutamic acid and one leucine residue.

From Table 15 it can be seen that of the various dipeptides that would be produced on breakdown of the five sequences, only one (Pro.Lys) was not detected. These results are in complete accord with the view that there is only one polypeptide chain in fraction *B*. All peptide spots present in detectable amounts have been taken into account and no peptides have been found that contradict this view. This would be almost impossible if there were two different chains in fraction *B* or if there was a significant amount of impurity.

SUMMARY

1. Partial hydrolysates of fraction *B* of oxidized insulin (Sanger, 1949*a*) have been fractionated by paper chromatography, and the structure of the resulting peptides determined.

2. It is concluded that the following amino-acid sequences are present in this fraction (abbreviations are given in Table 1, p. 463): Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly, Thr.Pro.Lys.Ala, Gly.Glu.Arg.Gly, Tyr.Leu.Val.$CySO_3H$.Gly and Ser.His.Leu.Val.Glu.Ala.

We wish to express our thanks to the Medical Research Council for a grant to cover the cost of the insulin and to Dr R. L. M. Synge for giving us details of the four-compartment ionophoresis cell prior to publication. One of us (H.T.) wishes to thank the British Council for a Scholarship.

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The Amino-acid Sequence in the Phenylalanyl Chain of Insulin

2. THE INVESTIGATION OF PEPTIDES FROM ENZYMIC HYDROLYSATES

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In the previous paper (Sanger & Tuppy, 1951) a large number of simple peptides in partial hydrolysates of fraction *B* of oxidized insulin were identified and several amino-acid sequences were deduced from them. It was not possible to determine the complete structure of the polypeptide chain, partly on account of the difficulty of fractionating the less polar peptides containing the aromatic amino-acids and partly because of the relatively great lability of certain bonds such as those involving the amino groups of the hydroxyamino-acids.

In the present paper are described investigations of the peptides produced on hydrolysis of fraction *B* with the proteolytic enzymes, pepsin, chymotrypsin and trypsin. These peptides, which are mostly larger than those present in the acid and alkaline hydrolysates, could be successfully fractionated by paper chromatography, and with the knowledge presented in the previous paper it was possible to determine the structure of most of them and to work out the complete sequence of fraction *B* and hence of the phenylalanyl chain of insulin.

The abbreviations, etc., defined in the previous paper (Sanger & Tuppy, 1951) will be used throughout and the peptides with reference numbers starting *B* 1, *B* 2, *B* 3, *B* 4, *B* 5 refer to those described in that paper.

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METHODS

Enzymes

Crystalline pig pepsin was obtained from Armour Laboratories. The chymotrypsin was prepared and several times recrystallized by Mr J. Lightbown of the National Institute for Medical Research, Mill Hill, London. The trypsin was a crystalline preparation from Armour Laboratories. It was recrystallized three times according to the method of Kunitz & Northrop (1936).

Hydrolyses

Experiment Bp (*pepsin*). Fraction *B* (30 mg.) was dissolved in 3 ml. 0·01 M-HCl. A solution of 0·3 mg. pepsin in 0·3 ml. 0·01 M-HCl was added, and the mixture incubated at 37° for 24 hr. After boiling to inactivate the pepsin, the solution was taken to dryness *in vacuo* to remove excess HCl and subjected to ionophoresis in the four-compartment cell of Synge described in the previous paper. This separated the hydrolysate into acid, neutral and basic fractions.

Experiment Bc (*chymotrypsin*). Fraction *B* (50 mg.) was dissolved in 5 ml. water and brought to pH 7·5–8·0 by the addition of $NaHCO_3$. To this was added 2·3 mg. chymotrypsin, the pH again adjusted and the solution incubated at 25° for 24 hr. It was then brought to about pH 3 by addition of HCl and taken to dryness in a desiccator. The hydrolysate was again separated into acidic, neutral and basic fractions by ionophoresis.

Experiment Bt (*trypsin*). Fraction *B* (25 mg.) was dissolved in 2·5 ml. water and the pH adjusted to 7·5 with $NaHCO_3$. Trypsin (1·2 mg.) dissolved in 1·2 ml. water was added and the mixture incubated at 37° for 24 hr. On acidification a

precipitate formed which was removed by centrifugation. It yielded on acid hydrolysis all the amino-acids that are present in fraction *B* and was not investigated further. The solution was then taken to dryness *in vacuo*.

Investigation of the peptides

The techniques used to investigate the peptides were the same as those described in the previous paper. Each fraction was studied on two-dimensional chromatograms using Whatman no. 4 or 3 filter papers. The latter is thicker and is useful when larger amounts of material are required. Butanol-acetic acid was found to be the most generally useful solvent for fractionating these large peptides, and this was usually preceded by cresol. Although in certain cases (e.g. Fig. 2) there were no apparent separations with the first solvent, it was found that much better results were obtained on two-dimensional than on one-dimensional chromatograms. Probably the first solvent removes traces of salt and other impurities and distributes each spot over a wider area.

With these larger peptides the amino-acids present were identified using two-dimensional chromatograms and *N*-terminal residues with the dinitrophenyl (DNP) technique. This was usually done both by identification of the free amino-acids and of the DNP-amino-acid present in a hydrolysate of the DNP-peptide. Due to the small amounts of peptides present it was not usually possible to identify the DNP-amino-acid completely on a number of different columns (Sanger, 1945), but it was usually put on a chromatogram that would distinguish it from other amino-acids likely to be present as terminal residues in the peptide.

Several of the peptides were subjected to further hydrolysis with a different enzyme. This was carried out in the following way: eluates of spots, usually from two chromatograms on Whatman no. 4 filter paper, were transferred to a polythene strip and taken to dryness in a desiccator. A solution (0·1 ml.) containing 0·1 mg. of the enzyme adjusted to a suitable pH was then added. The pepsin was dissolved in 0·01 N-HCl and the trypsin was dissolved in water and adjusted to pH 7·5 with $NaHCO_3$. The drops were then transferred to capillary tubes and incubated at 37° for 24 hr. After putting on to polythene strips again they were acidified, taken to dryness and fractionated on a suitable two-dimensional chromatogram.

RESULTS

Experiment Bp (pepsin)

The results obtained with the neutral and basic fractions of the hydrolysate are recorded in Figs. 1 and 2 and Table 1. The acidic fraction was investigated in three of the experiments. No significant amounts of peptides were obtained in two cases, but in the third peptides 1, 2, 4, 11, 12 and 13 (Table 1) could be detected. The different results were probably due to slight differences of pH in the middle compartment during ionophoresis.

Spot 1. Contains traces of Val.Glu which can be separated on a phenol chromatogram.

Eluates of spot 1 from two phenol/butanol-acetic acid chromatograms were hydrolysed 48 hr. in 10 N-HCl at 37° and the hydrolysate fractionated on a phenol/butanol-acetic acid chromatogram. Besides free amino-acids the peptides Glu.Ala (×), Val.Glu (× ×) and unchanged tripeptide (× × ×) were identified. This demonstrates that its structure must be Val.Glu.Ala. It is the same as spot $B1\gamma10$ and has the same R_F values on phenol and cresol. The Val.Glu present has the same R_F value as peptide $B1\gamma9$. This indicates that they are probably not present as amides but with the γ-carboxyl group of glutamic acid free.

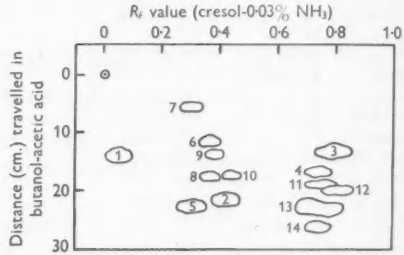

Fig. 1. Chromatogram of the neutral fraction of the peptic hydrolysate (*Bp*). In Figs. 1–6 the fraction was applied in the position marked by the small dotted circle, and the chromatogram run first in the direction represented horizontally, which except in Figs. 2 and 4 was the longer dimension of the filter paper. In each case both solvents were run for one length of the filter paper only (see Table 1).

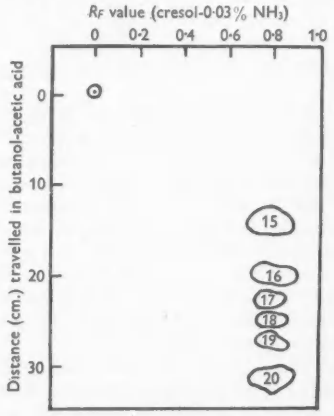

Fig. 2. Chromatogram of the basic fraction of the peptic hydrolysate (*Bp*). The chromatogram was run first down the short dimension of the filter paper with cresol-0·03% NH_3 (see Table 1).

Spot 2. This is not the same as spot $B2\beta15$, which contains the same amino-acids, but has leucine as the *N*-terminal residue.

Eluates from two chromatograms were hydrolysed 3 days in 10 N-HCl at 37° and fractionated on a cresol/butanol-acetic acid chromatogram. Besides free amino-acids and small amounts of tripeptides, the dipeptides Val.Glu and Ala.Leu were identified and were present in considerable amounts. From this result it is concluded that the tetrapeptide is Val.Glu.Ala.Leu.

Spot 3. The results in Table 1 indicate that phenylalanine is the *N*-terminal residue. This was confirmed by showing

Table 1. *Peptides from peptic hydrolysate (Bp)*

(Amino-acids formed after hydrolysis of the peptide (*H*) or of the DNP-peptide (*D*).)

Spot no. (Figs. 1 and 2)	*H* or *D*	Amino-acids formed after hydrolysis														
		$CySO_3H$	Asp	Glu	Ser	Gly	Thr	Ala	Lys	Tyr	TyrX	His	Val	Leu	Arg	Phe	Pro
1	*H*	—	—	× × ×	—	—	—	× × ×	—	—	—	—	× × ×	—	—	—	—
	D	—	—	× ×	—	—	—	× ×	—	—	—	—	—	—	—	—	—
2	*H*	—	—	× ×	—	—	—	× ×	—	—	—	—	× ×	× ×	—	—	—
	D	—	—	× ×	—	—	—	× ×	—	—	—	—	?	× ×	—	—	—
3	*H*	× ×	× ×	× ×	× ×	× ×	—	?	—	—	—	× × ×	× ×	× × ×	—	× ×	—
	D	×	×	×	×	× ×	—	?	—	—	—	× *	×	× ×	—	?	—
4	*H*	× ×	—	× ×	—	× × ×	—	—	—	—	—	—	× ×	× ×	× ×	× ×	—
5	*H*	—	—	×	—	—	—	—	—	—	—	—	×	×	—	—	—
6	*H*	?	—	?	—	×	—	—	—	—	—	—	?	?	?	—	—
7	*H*	—	?	?	—	—	—	—	—	—	—	—	?	—	—	—	—
8	*H*	—	×	×	—	—	—	—	—	—	—	—	×	—	—	?	—
9	*H*	—	—	—	—	—	—	—	—	×	—	—	—	—	—	—	—
10	*H*	—	—	—	—	—	—	—	—	—	× ×	—	—	—	—	—	—
11	*H*	× ×	—	× ×	—	× × ×	—	—	—	—	—	—	× ×	× ×	× ×	× × ×	—
12	*H*	× ×	—	× ×	—	× × ×	—	?	—	× ×	—	—	× ×	× ×	× ×	× ×	—
13	*H*	× ×	—	× ×	—	× × ×	—	(× ×)	—	(×)	—	—	× ×	× ×	× ×	× ×	—
14	*H*	—	—	—	—	—	—	×	—	×	—	—	—	× ×	—	× ×	—
15	*H*	—	—	—	—	—	× ×	× ×	× ×	× ×	—	—	—	—	—	—	—
	D	—	—	—	—	—	× ×	× ×	× *	—	—	—	—	—	—	—	× ×
16	*H*	—	—	—	—	—	× × ×	× × ×	× × ×	—	× ×	—	—	—	—	—	× ×
	D	—	—	—	—	—	× ×	× ×	× *	—	—	—	—	—	—	—	× ×
17	*H*	× ×	—	—	× ×	× ×	—	—	—	—	—	× × ×	—	× × ×	—	—	×
18	*H*	× ×	× ×	× ×	× ×	× × ×	—	—	—	—	—	× × ×	× ×	× × ×	—	×	—
19	*H*	×	×	×	×	×	?	×	×	—	?	×	×	×	—	×	?
20	*H*	×	?	×	×	×	?	×	×	—	?	×	?	×	—	×	?

* DNP derivative.

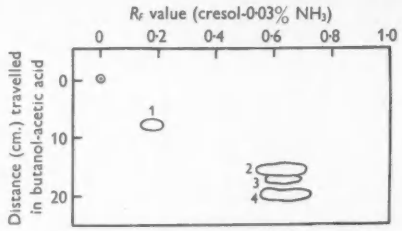

Fig. 3. Chromatogram of the neutral fraction of the chymotryptic hydrolysate (*Bc*) (see Table 2).

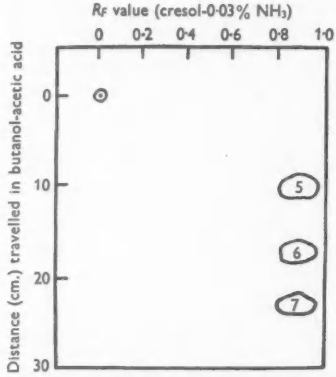

Fig. 4. Chromatogram of the basic fraction of the chymotryptic hydrolysate (*Bc*), run first down the short side of the paper with cresol-0·03% NH_3 (see Table 2).

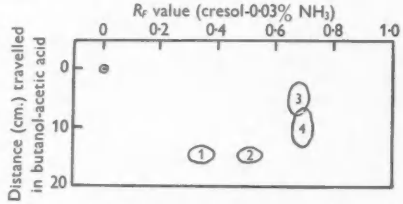

Fig. 5. Chromatogram of peptic hydrolysate of fraction *Bc*2 (see Table 2).

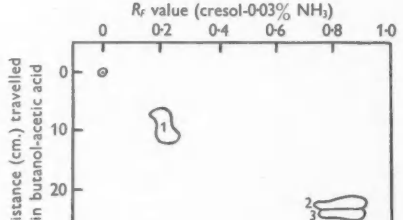

Fig. 6. Chromatogram of the tryptic hydrolysate (*Bt*) (see Table 2).

Table 2. *Peptides from chymotryptic (Bc) and tryptic (Bt) hydrolysates*

Spot no. (Figs. 3, 4, 5 and 6)	Amino-acids formed after hydrolysis																				
	$CySO_3H$	Asp	Glu	Ser	Gly	Thr	Ala	Lys	Tyr	TyrX	His	Val	Leu	Arg	Phe	Pro
*Bc*1	× ×	—	×	—	×	—	—	—	—	—	—	×	×	×	—	—
*Bc*1 (DNP)	× ×	—	×	—	×	—	—	—	—	—	—	×	—	×	—	—
*Bc*2		×	×	×	×	—	×	—	(?)	(×)	×	×	×	—	×	—
*Bc*3		×	×	×	×	—	×	—	(×)	(××)	×	×	×	—	×	—
*Bc*4		×	×	×	×	—	×	—	—	—	×	×	×	—	×	—
*Bc*5		×	×	×	×	—	×	×	—	—	×	×	×	×	×	—
*Bc*6		—	—	—	—	×	×	×	×	—	—	—	—	—	—	×
*Bc*7		—	—	—	—	×	×	×	—	×	—	—	—	—	—	×
*Bc*2*p*1		—	×	—	—	—	×	—	×	—	—	×	×	—	—	—
*Bc*2*p*2		—	×	—	—	—	×	—	—	—	—	—	×	—	—	—
*Bc*2*p*3		—	×	×	×	—	×	—	×	—	—	×	×	—	×	—
*Bc*2*p*4		—	×	×	×	—	×	—	—	—	×	×	×	—	×	—
*Bt*1		—	—	—	—	—	×	×	×	—	—	—	—	—	—	×
*Bt*2		—	—	—	×	×	×	×	×	—	—	—	—	—	×	×
*Bt*3		—	—	—	×	—	—	×	—	×	—	—	—	—	×	×
*Bt*2*p*1		—	—	—	—	×	—	×	×	—	—	—	—	—	—	×
*Bt*2*p*2		—	—	—	×	—	—	—	—	—	—	—	—	—	×	—

that the DNP-amino-acid produced on hydrolysis of the DNP-peptide moved at the same R value as DNP-phenylalanine on an ethanol-ligroin column (Sanger, 1945). This distinguishes it from the DNP derivatives of the other amino-acids present in spot 3, except possibly glycine.

Spot 4. On hydrolysis of the DNP derivative, a DNP-amino-acid was produced that was identified as DNP-leucine or DNP-valine. Chromatography of the remaining amino-acids with benzyl alcohol showed strong spots for valine and phenylalanine and only a weak leucine spot, indicating that leucine was the N-terminal residue.

Eluates from two chromatograms were subjected to hydrolysis with trypsin. The hydrolysate was fractionated on a half sheet of Whatman no. 1 filter paper using cresol-0·03 % NH_3/butanol-acetic acid. Only two spots were present, whose R_F values and compositions are shown in Table 3. *Bp*4*t*2 is the same as spot $B1\gamma12a$ (Gly.Phe). Further results with this peptide are given in connexion with peptide *Bc*4.

Spot 1. When a different preparation of chymotrypsin (obtained by activation of chymotrypsinogen with trypsin) was used this spot was much stronger. It was also present in hydrolysates obtained by the use of unrecrystallized trypsin, and thus appears to be produced by the combined action of chymotrypsin and trypsin on fraction B. The small amount present in this experiment may be due to traces of trypsin in the preparation.

Spot 2. This sometimes contains varying amounts of tyrosine and TyrX.

A hydrolysate of the DNP derivative was put on a benzyl alcohol chromatogram. It gave a weak phenylalanine spot but strong spots for leucine and valine, showing phenylalanine to be the N-terminal residue.

The eluates of spot 2 from two chromatograms were treated with pepsin and the hydrolysate fractionated on a half sheet of Whatman no. 1 filter paper using cresol/butanol-acetic acid. The results are shown in Fig. 5 and

Table 3. *Tryptic hydrolysis of fractions Bp* 4 *and Bc* 4

Fraction	R_F value (cresol-0·03 % NH_3)	Distance in butanol-acetic acid (cm.)	Strength	Composition
*Bp*4*t*1	0·19	5·6	× × ×	$CySO_3H$, Glu, Gly, Val, Leu, Arg
*Bp*4*t*2	0·66	14·4	× × ×	Gly, Phe
*Bc*4*t*1	0·21	6·9	× ×	$CySO_3H$, Glu, Gly, Val, Leu, Arg
*Bc*4*t*2	0·87	20·8	× ×	Gly, Phe

Spot 5. A small amount of Leu.Val.Glu identical with peptide $B2\beta14$.

Spot 7. Trace of Val.Asp.Glu.

Spot 8. Phe.Val.Asp.Glu. Peptides 7 and 8 probably contain glutamine and asparagine residues (Sanger, 1949*b*) and are therefore not identical with the corresponding peptides from the acid hydrolysates ($B1\beta10$, $B1\beta12$ respectively).

Spots 9 *and* 10. Free tyrosine and TyrX respectively.

Spots 11 *and* 12. In most experiments these two spots were not clearly separated and their composition varied considerably. The results are from a single experiment. Usually they contain free phenylalanine and leucine and at least two peptides, which contain arginine but no aspartic acid and have variable amounts of tyrosine.

Spot 13. Contains arginine but no aspartic acid. The amounts of tyrosine and alanine varied somewhat from one experiment to another. Alanine varies independently of the other amino-acids so is probably part of a low-molecular peptide (e.g. Ala.Leu). Tyrosine is probably connected with the larger peptide.

Spot 14. Probably a mixture of fast-moving small peptides.

Spot 15. The presence of tyrosine as an N-terminal residue was confirmed by showing that hydrolysis of the DNP derivative gave rise to a DNP-amino-acid that moved on a $CHCl_3$ column at the same rate as ON-bis-DNP-tyrosine. This distinguishes it from DNP-threonine.

Spot 16. The same as 15, but containing TyrX instead of tyrosine.

Spot 18. The same as spot 3 (neutral fraction).

Experiment Bc (chymotrypsin)

The results obtained with the chymotryptic hydrolysate are given in Figs. 3 and 4 and Table 2.

Table 2. Spots *Bc*2*p*1 and *Bc*2*p*3 are apparently the same as *Bp*2 and *Bp*3 respectively, which are found in the peptic hydrolysate.

Spot 3. This contains varying amounts of tyrosine and TyrX, showing that it is impure.

A hydrolysate of the DNP derivative was shown on a benzyl alcohol chromatogram to contain a trace of phenylalanine and large amounts of leucine and valine, indicating that phenylalanine was the N-terminal residue.

A peptic hydrolysate gave a chromatogram similar to that obtained with spot *Bc*2 (Fig. 5). Spots corresponding to *Bc*2*p*1 and *Bc*2*p*2 were present but only one other large spot in a position corresponding to that of *Bc*2*p*3 and *Bc*2*p*4. This contained all the amino-acids of the original material, and was thus probably a mixture of *Bc*2*p*3 and *Bc*2*p*4 with unhydrolysed *Bc*3.

Table 4. *Amino-acid composition of peptides Bp* 4*t*2 *and Bc* 4*t*2

(Results expressed as μmol. amino-acid in peptide originating from 10 mg. fraction *B*.)

Peptide	Glycine	Phenylalanine
*Bp*4*t*2	0·3	0·3
*Bc*4*t*2	0·1	0·23

Spot 4. An eluate from one chromatogram was hydrolysed with trypsin and fractionated on cresol-0·03 %NH_3/butanol-acetic acid. Two spots were obtained and the results are shown in Table 3 together with those for the corresponding spots from peptide *Bp*4. Peptides *Bp*4*t*1 and *Bc*4*t*1 are apparently identical. However, peptide *Bc*4*t*2 is different from *Bp*4*t*2 since it moves considerably faster with both solvents. It gives a yellow colour with ninhydrin suggesting that glycine is the N-terminal residue. In Table 4 are shown

the results of an experiment in which the glycine and phenylalanine in the two peptides were estimated by semi-quantitative paper chromatography. Known quantities of authentic amino-acid were run in parallel on a chromatogram and the strength of the ninhydrin spots compared visually. Whereas *Bp*4*t*2 contains the two amino-acids in equal proportions, *Bc*4*t*2 has two phenylalanine residues to one glycine residue.

Experiment Bt (trypsin)

The soluble material from the tryptic hydrolysate was fractionated directly without any preliminary group separation. Fig. 6 shows a typical chromatogram. Only three significant spots were present, and their composition is shown in Table 2.

Spot 1. Contained only alanine, and its R_F values and strong ninhydrin reaction indicated that it was the free amino-acid.

Spot 2. The slower moving parts of the spot frequently contained traces of glutamic acid, alanine, valine and leucine, which do not appear to be an integral part of the peptide. It gave a yellow colour with ninhydrin, suggesting that glycine or threonine was the *N*-terminal residue. Hydrolysis of the DNP derivative gave a DNP-amino-acid that moved on a $CHCl_3$ column at the same rate as DNP-glycine (or bis-DNP-lysine or *ON*-bis-DNP-tyrosine) and the spot for free glycine was considerably weaker than in the hydrolysate of the untreated peptide. It was thus concluded that glycine is the *N*-terminal residue.

Eluates from two chromatograms were subjected to hydrolysis with pepsin, and the hydrolysate was fractionated on a collidine/benzyl alcohol chromatogram. Only two spots were identified (see Table 2). *Bt*2*p*1 had $R_F = 0{\cdot}35$ in collidine and had travelled 7 cm. in benzyl alcohol. *Bt*2*p*2 had $R_F = 0{\cdot}46$ and had travelled 11·5 cm. It is not clear from these results whether peptide *Bt*2*p*2 is identical with *Bp*4*t*2 ([Gly, Phe]) or with *Bc*4*t*2 ([Gly, Phe, Phe]).

Spot 3. The same as spot 2, but containing TyrX instead of tyrosine.

DISCUSSION

With the rather larger peptides present in these enzymic hydrolysates, it was important to know if certain spots contained only one peptide or whether they could be mixtures. Although direct proof of homogeneity is difficult the following factors could be taken into account in assessing the purity of a spot:

(1) Constant amino-acid composition over different parts of the spot and of the same spot from different chromatograms.

(2) Comparison of the R_F value of the spot with that to be expected if all the amino-acids were present in a single peptide and with the R_F values of possible components of a mixture (see Martin, 1949).

(3) The presence of only one *N*-terminal residue is probably the best evidence of purity, since it is unlikely that two different peptides would have the same *N*-terminal residue.

(4) Consideration of the sequences previously shown to be present in fraction *B* would often indicate whether a given spot was likely to be pure or impure.

Structure of the peptides

In the previous paper the sequences Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly, Thr.Pro.Lys.Ala, Gly.Glu.Arg.Gly, Tyr.Leu.Val.$CySO_3H$.Gly and Ser.His.Leu.Val.Glu.Ala were shown to be present in fraction *B*. These sequences account for all the residues present except two phenylalanine, one tyrosine and possibly one leucine and one glutamic acid residue. Other peptides present were Ala.Leu, Gly.Phe and Ala.[Tyr, Leu].

Peptide *Bp* 3 contains aspartic acid and hence at least part of the *N*-terminal sequence Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly, in which the only aspartic acid residue in fraction *B* is present. It has a phenylalanine *N*-terminal residue and contains all the amino-acids present in this sequence and also serine. There is only one serine residue in fraction *B* and this is present in the sequence Ser.His.Leu.Val.Glu.Ala, so that this establishes a connexion between these two sequences. None of the other sequences above could come between the glycine and serine residues, since peptide *Bp* 3 contains no threonine, proline, lysine, arginine or tyrosine. The possibility that a phenylalanine, leucine or glutamic acid residue may come between the glycine and serine should be considered since all of these residues in fraction *B* have not yet been accounted for. Peptide *Bp* 17, which has cysteic acid, glycine, serine, histidine and leucine but no valine, must contain parts of both the above two sequences. It contains no phenylalanine or glutamic acid, so that neither of these two can be between the glycine and serine residue. It is very unlikely that there is a leucine residue here since Gly.Leu has never been observed, whereas the similar Gly.Phe was detected. Also it will be shown below that all the leucine residues of fraction *B* can be accounted for elsewhere. Thus it may be concluded that the *N*-terminal peptide sequence of fraction *B* is Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly.Ser.His.Leu.Val.Glu.Ala. The Gly.Ser bond would be extremely labile and was therefore not found intact in any of the peptides obtained from acid or alkaline hydrolysis. The exact structure of peptide *Bp* 3 cannot be deduced from the present results, but it most probably contains the first eleven residues of the above sequence, since the presence of Val.Glu.Ala.Leu (*Bp* 2) shows that the Leu.Val bond is split by pepsin. It must contain at least the first nine residues as it has serine but not more than thirteen residues as alanine is absent. Peptide *Bp* 17 is most probably His.Leu.$CySO_3H$.Gly.Ser.His.Leu. This is suggested by the presence of Phe.Val.Asp.Glu (*Bp* 8) and Val.Asp.Glu (*Bp* 7) in the peptic hydrolysate, which demonstrates a lability of the Glu.His bond to pepsin.

Peptide *Bp* 2 was shown by partial hydrolysis to be Val.Glu.Ala.Leu. This must be joined to the above

N-terminal sequence, since the other alanine residue of fraction *B* is in the form Lys.Ala. Ala.Leu (*B* 1δ11) was found in the acid hydrolysate. Further evidence for this *N*-terminal sequence is derived from peptides *Bc* 2 and *Bc* 3. These contain the amino-acids of the above sequence and a phenylalanyl *N*-terminal residue. They also have alanine and varying amounts of tyrosine, which shows they are longer than *Bp* 3. On hydrolysis with pepsin *Bc* 2 (Fig. 5, Table 2) gives a peptide (*Bc* 2*p*3) which appears to be identical with *Bp* 3 and also Val.Glu.Ala.Leu (*Bc* 2*p*1) identical with *Bp* 2. It also gives a peptide [Glu, Ala, Tyr, Val, Leu] (*Bc* 2*p*2). This can only be Val.Glu.Ala.Leu.Tyr and establishes the position of tyrosine on the *N*-terminal sequence, which must therefore be Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.-Gly.Ser.His.Leu.Val.Glu.Ala.Leu.Tyr. This will be referred to as sequence 1. Further evidence for this structure comes from peptide *B* 3β9, which must be Ala.Leu.Tyr. Leu.Tyr is probably present in spot *B* 3β10. The indefinite result with the DNP method suggests both Leu.Tyr and Tyr.Leu are present.

The variation in the amounts of tyrosine in spots *Bc* 2 and *Bc* 3 suggests that they are probably mixtures of closely related peptides containing the *N*-terminal sequence and varying amounts of leucine, tyrosine and TyrX. It is probable that pepsin can split the bonds on both sides of this tyrosine residue.

Fraction *B* contains three glycine residues, one of which is present in the above sequence; others are in the two sequences Tyr.Leu.Val.$CySO_3H$.Gly and Gly.Glu.Arg.Gly, which must therefore be parts of the same sequence. Thus the sequence in fraction *B* must be Tyr.Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly. The only other peptide containing glycine is Gly.Phe (*B* 1γ12*a*) which was obtained from the acid hydrolysate. It can only be combined to the *C*-terminal glycine of the above octapeptide establishing the sequence Tyr.Leu.Val.$CySO_3H$.-Gly.Glu.Arg.Gly.Phe.

A number of peptides from the pepsin and chymotrypsin hydrolysates offer further evidence for this sequence. Peptide *Bp* 4 contains arginine as well as valine and cysteic acid and has leucine as the *N*-terminal residue, thus demonstrating the connexion between the two sequences Tyr.Leu.Val.$CySO_3H$.-Gly and Gly.Glu.Arg.Gly. On hydrolysis with trypsin two peptides are produced. One (*Bp* 4*t*2) is Gly.Phe, the other (*Bp* 4*t*1) contains all the amino-acids of *Bp* 4 except phenylalanine. It is probably identical with peptide *Bc* 1, which was very strong when what appears to be a mixture of trypsin and chymotrypsin was used for hydrolysis. It contains a leucine *N*-terminal residue and can only be Leu.Val.$CySO_3H$.Gly.Glu.Arg; *Bp* 4 must be Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe, the trypsin splitting after the arginine residue.

Peptide *Bc* 4 is also derived from this sequence and contains the same amino-acids as *Bp* 4. On hydrolysis with trypsin it gives the same hexapeptide Leu.Val.$CySO_3H$.Gly.Glu.Arg (*Bc* 4*t*1), but instead of Gly.Phe it gives a spot (*Bc* 4*t*2) moving considerably faster, but containing only these two amino-acids. A semi-quantitative analysis (Table 4) indicated that the ratio of glycine to phenylalanine in this peptide is 1:2, and the only possible conclusion is that it is Gly.Phe.Phe and that peptide *Bc* 4 is Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe.-Phe. The sequence in fraction *B* is thus Tyr.Leu.-Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe.Phe, which will be referred to as sequence 2.

Spots *Bp* 11, *Bp* 12, *Bp* 13 are probably mixtures of various peptides containing this sequence, with varying amounts of tyrosine, alanine, leucine and phenylalanine. On both sides of this sequence there are several bonds that may be split by pepsin, so that one would expect to find a large number of closely related peptides. Peptide *Bp* 6 is probably a trace of Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly. It is unlikely that pepsin would split the bond next to arginine, whereas it would be expected to split the bond before the phenylalanine residue.

The only other sequence deduced from the acid hydrolysis experiments that we have not so far considered is Thr.Pro.Lys.Ala, which is present as the tetrapeptide in spot *Bc* 5. Peptides *Bc* 6, *Bc* 7, *Bp* 15, *Bp* 16 all contain the same four residues together with tyrosine or TyrX which is their *N*-terminal residue. Their structure must therefore be Tyr.Thr.Pro.-Lys.Ala. Peptide *Bt* 2 contains threonine, proline and lysine which are unique to this sequence and also phenylalanine, which must be derived from the *C*-terminal end of sequence 2, thus establishing a connexion between these two sequences. Peptide *Bt* 2 has a glycyl *N*-terminal residue and must therefore be Gly.Phe.Phe.Tyr.Thr.Pro.Lys. On hydrolysis with pepsin it gives Tyr.Thr.Pro.Lys (*Bt* 2*p*1) and a peptide (*Bt* 2*p*2) containing glycine and phenylalanine, probably Gly.Phe.Phe. Sequence 2 must now be Tyr.Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.-Phe.Phe.Tyr.Thr.Pro.Lys.Ala.

Structure of fraction B

Fraction *B* contains only two tyrosine residues. One is accounted for within sequence 2, the other is present both as the *C*-terminal residue of sequence 1 and as the *N*-terminal residue in sequence 2. The two sequences must therefore have this tyrosine residue in common, and the structure of fraction *B* must be Phe.Val.Asp.Glu.His.Leu.$CySO_3H$.Gly.Ser.-His.Leu.Val.Glu.Ala.Leu.Tyr.Leu.Val.$CySO_3H$.-Gly.Glu.Arg.Gly.Phe.Phe.Tyr.Thr.Pro.Lys.Ala. This structure has alanine as a *C*-terminal residue. This confirms the results of Lens (1949), who studied the action of carboxypeptidases on insulin and

showed that alanine was present as a *C*-terminal residue. Fromageot, Jutisz, Meyer & Pénasse (1950) and Chibnall & Rees (1951) have also independently identified alanine as the *C*-terminal residue in insulin using their respective methods of reduction to β-amino alcohols. The liberation of free alanine (*Bt* 1) by the action of trypsin on fraction *B* also supports this conclusion. Studies with synthetic peptides have shown that trypsin splits bonds involving the carboxyl groups of arginine or lysine. It will therefore be expected to split the bond before the alanine residue, but not that after it. Free alanine could only be produced if it is already present as a *C*-terminal residue.

Table 5, which summarizes the foregoing discussion, gives the structure of fraction *B* and of the various peptides that have played a major part in the elucidation of the sequence. The residues have been numbered for convenience in the subsequent discussion. The phenylalanyl chains of insulin have the same structure as fraction *B* except that the $CySO_3H$ residues are replaced by CyS (half-cystine) residues.

The above sequence explains the difficulties experienced in fractionating the fast-moving aromatic peptides (Sanger & Tuppy, 1951). A large proportion of the least polar residues are concentrated together in the two sequences Leu.Tyr.Leu.-Val and Phe.Phe.Tyr, which can be expected to give rise on partial hydrolysis to a large number of closely related peptides having similar R_F values. The difficulty is increased by the presence of TyrX.

Further evidence for the presence of the sequence Phe.Phe was obtained from a chromatogram of an aromatic fraction which was prepared from a 9-day low-temperature acid hydrolysate of insulin by adsorption on charcoal. A peptide spot was identified which had $R_F = 0{\cdot}61$ on collidine and 0·54 on benzyl alcohol and contained only phenylalanine. The free amino-acid had $R_F = 0{\cdot}40$ on collidine, 0·30 on benzyl alcohol on the same chromatogram. The only possible explanation is that the faster moving spot was Phe.Phe.

Since synthetic reactions have been shown to take place in the presence of proteolytic enzymes, the possibility that some of the peptides identified could have been formed by rearrangement of peptide bonds during the course of hydrolysis should be considered. While such a rearrangement would seem rather unlikely, we have attempted as far as possible to obtain evidence for the presence of each bond, either by identifying it in a peptide obtained by acid hydrolysis or else by identifying it in peptides produced by the action of at least two different enzymes. It is extremely unlikely that the same bond would be synthesized in both cases. The only bond to which this does not apply is the Phe.Tyr bond (positions 25, 26). This was intact only in the peptide *Bt* 2, but it

Table 5. *The structure of fraction B of oxidized insulin*

Sequences deduced from lower peptides (Sanger & Tuppy, 1951)																															Thr.Pro.Lys.Ala
																															(*Bt* 1)
																															Ala

is very improbable that such a bond would be formed by the action of trypsin, since the specificity for synthetic reactions should be similar to that for hydrolytic reactions. Moreover, if any extensive rearrangements had occurred it would almost certainly have been impossible to interpret the results in terms of a single amino-acid sequence for fraction *B*.

The question of location of the amide groups is not completely settled by the present work. The results with the *N*-terminal residues (Sanger, 1949*b*) showed that the aspartic and glutamic acid residues in positions 3 and 4 are in the form of amides, since several different DNP-peptides containing the four residues phenylalanine, valine, aspartic and glutamic acids were detected. According to the amide analysis of fraction *A* (Sanger, 1949*a*) and of insulin (Rees, 1946), there would appear to be only two amide groups in fraction *B*, so that the other two glutamic acid residues (positions 13, 21) would be expected to have free γ-carboxyl groups. If an amide residue is present in a protein it would be expected to be found still intact in most peptides derived from an enzymic hydrolysate, but with the amide group removed in the corresponding peptides from an acid hydrolysate. The peptides Val.Glu.Ala (*Bp* 1), Val.Glu (also in *Bp* 1) and Leu.Val.Glu (*Bp* 5) derived from the peptic hydrolysate all have the same R_F values as the corresponding peptides from the acid hydrolysates (*B* 1γ10, *B* 1γ9, *B* 2β14 respectively), which suggests that they are not present as amides and that the γ-carboxyl group of the glutamic acid residue in position 13 is free in insulin. No such evidence is available for the glutamic acid residue in position 21, but the fact that peptide *Bp* 4 (Leu.Val.$CySO_3H$.Gly.Glu.Arg.Gly.Phe) is sometimes found in the acidic fraction from the ionophoresis suggests that the γ-carboxyl group of the glutamic acid residue is also free here.

In recent years several hypotheses of protein structure have been advanced which are based on the assumption of some type of periodic arrangement of amino-acids along the polypeptide chains. It is tempting to assume that this arrangement, which is determined by the unknown mechanisms of protein synthesis, may be rather simple and may follow certain easily discernible principles. An examination of the structure of fraction *B*, however, fails to reveal any simple periodic arrangement of the residues, nor is it possible to formulate any general principles which might govern the order of amino-acids along the protein chains. It may be that when much more extensive data are available such principles may become apparent; meanwhile two interesting features of the structure, which may or may not be significant, seem worth noting.

The presence of three aromatic residues joined together in the sequence Phe.Phe.Tyr is of interest and suggests that there may be a certain tendency for the less polar residues to be grouped together. The sequence Leu.Tyr.Leu.Val is another similar grouping. Such an arrangement might be of importance in connexion with the physical or biological properties of the insulin.

In the structure given for fraction *B* there are three dipeptide sequences which occur twice in the chain. These are His.Leu (positions 5–6, 10–11), $CySO_3H$.Gly (positions 7–8, 19–20) and Leu.Val (positions 11–12, 17–18). This is more than one would expect from a mere random arrangement and might suggest a certain limitation in the mechanisms by which proteins are synthesized or it might indicate that dipeptide units are the essential building stones of proteins. It is interesting to note that one of these sequences (CyS.Gly) also occurs in glutathione.

It is difficult to say if either of these features will prove to have general significance. They certainly would not be expected if the arrangement were entirely random, but it is probably more pertinent to consider how many of the possible arrangements of the amino-acids found in fraction *B* would not show some interesting or unusual features of this type.

Action of the proteolytic enzymes on fraction B

Since the purpose of this investigation was primarily to determine the structure of fraction *B*, all the precautions necessary for a careful study of the specificity of proteolytic enzymes have not been taken, nor has the exact structure of all the peptides found been determined. Although it is believed that all the enzymes used are essentially homogeneous, no rigorous test of purity was used, nor was it possible to control accurately the pH in the very small volumes of the tryptic and chymotryptic hydrolysis mixtures. However, certain conclusions concerning the action of the enzymes can profitably be drawn, since this is the first time that such a large polypeptide has been studied. In Table 5 are indicated the various linkages that are split by the three enzymes used. It will be seen that the specificities of trypsin and chymotrypsin, which were defined by Bergmann and his associates using synthetic substrates, are almost completely applicable to this large polypeptide and thus probably to proteins also.

The only two bonds that are split to any extent by trypsin are those involving the carboxyl groups of the arginine and lysine residues. An exactly similar specificity was found by Bergmann, Fruton & Pollok (1939) and by Hofmann & Bergmann (1939) using synthetic substrates.

According to Bergmann & Fruton (1937) chymotrypsin splits those bonds involving the carboxyl groups of the aromatic residues phenylalanine and

tyrosine. A typical substrate was *N*-carbobenzyloxytyrosylglycine amide. In fraction *B* three of the aromatic residues are grouped together in the sequence Phe.Phe.Tyr. Since chymotrypsin does not split bonds which are adjacent to a free carboxyl group, the manner in which it would split this sequence would be expected to be rather complex. The present results indicate that the main splitting takes place after the two tyrosine residues (positions 16, 26) and after the third phenylalanine residue (position 25). The small amount of tyrosine in peptide *Bc* 2 suggests that some splitting of the Leu.Tyr bond (positions 15, 16) also occurs.

Pepsin appears to have a considerably wider range of specificity than the other two enzymes. Fruton & Bergmann (1939) showed that bonds involving the amino groups of the aromatic residues were readily split in synthetic substrates. Harington & Pitt-Rivers (1944) demonstrated that Tyr.CySH is also hydrolysed. Here the bond involves the carboxyl group of the tyrosine residue. The present results also suggest that splitting takes place on both sides of the aromatic residues, though here again the situation is rather complex, since in the two sequences Ala.Leu.Tyr.Leu.Val and Phe.Phe.Tyr most of the bonds would be susceptible to hydrolysis. It does not, however, appear that the specificity is limited to bonds adjacent to aromatic residues. Thus the Leu.-Val bond (positions 11, 12) is quite strongly hydrolysed. This is evident since Val.Glu.Ala.Leu (*Bp* 2) is present in considerable amounts. Val.Glu.Ala (*Bp* 1) is also present indicating that the Ala.Leu bond is also broken. Another unexpected finding was that the Glu.His bond (positions 4, 5) is broken. This was evident from the presence of Phe.Val.Asp.Glu (*Bp* 8) and of Val.Asp.Glu (*Bp* 7) in the hydrolysate. As already mentioned, the glutamic acid here is probably in the form of an amide so that the actual link split is glutaminylhistidine. Apart from this, it could be said that pepsin splits those bonds adjacent to tyrosine, phenylalanine and leucine residues. It would appear to have a specificity for the more fat-soluble parts of the peptide chain. The finding of a rather wide specificity is in agreement with the results of Desnuelle, Rovery & Bonjour (1950), who studied the *N*-terminal residues liberated on treatment of ovalbumin and horse globin with pepsin.

SUMMARY

1. Fraction *B* of oxidized insulin was subjected to hydrolysis by pepsin, trypsin and chymotrypsin. The resulting peptides were fractionated by paper chromatography and their structure was investigated.

2. It is concluded that the structure of the phenylalanyl chains of insulin is Phe.Val.Asp-($-NH_2$).Glu($-NH_2$).His.Leu.CyS.Gly.Ser.His.-Leu.Val.Glu.Ala.Leu.Tyr.Leu.Val.CyS.-Gly.Glu.Arg.Gly.Phe.Phe.Tyr.Thr.Pro.Lys.-Ala.

We wish to express our thanks to Mr J. Lightbown for a gift of chymotrypsin, and to the Medical Research Council for a grant to cover the cost of the insulin. One of us (H.T.) wishes to thank the British Council for a scholarship.

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Cholinesterase Inhibition *in vitro* by *OO*-Diethyl *O*-*p*-nitrophenyl Thiophosphate (Parathion, E 605)

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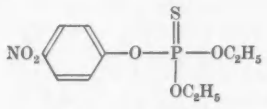
OO-Diethyl *O*-*p*-nitrophenyl thiophosphate (I), generally known as parathion or E 605, is an insecticide which is of value against a variety of crop pests. Its mammalian toxicity is usually ascribed to its power of inhibiting cholinesterase, and this is also possibly its mechanism of insecticidal action. The *in vitro* inhibition of the enzyme was first studied by DuBois, Doull, Salerno & Coon (1949), who found that with their experimental conditions a concentration of $1{\cdot}2 \times 10^{-6}$ M was necessary to produce 50 % inhibition of the enzyme; they therefore regarded it as a strong *in vitro* inhibitor. Later, Aldridge (1950) showed that a concentration of the same order was required to inhibit red-cell cholinesterase.

Hestrin (1949) has described a chemical method for the estimation of acetylcholine, which has been used recently by Wilson & Bergman (1950) to determine cholinesterase activity. We have employed a modification of this method to assess the inhibition produced by samples of parathion and analogous compounds from different sources, and the wide variation in the results obtained has been related to the purity of the samples.



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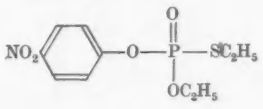

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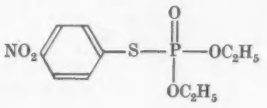

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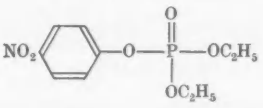

(IV)

EXPERIMENTAL

Reagents

Phosphate saline. This corresponds to a Krebs-Ringer phosphate solution without calcium.

Acetylcholine solution. A stock 0·1 M solution of acetylcholine chloride was prepared in 0·001 M-acetate buffer (pH 4·5) and stored in the refrigerator. When required, 3 ml. of this solution were diluted to 100 ml. with phosphate saline and brought to 37° in a constant temperature bath.

Alkaline hydroxylamine reagent. A 20 % (w/v) solution of hydroxylamine hydrochloride was prepared and stored in the refrigerator; it was discarded after 2 weeks. When the reagent was required the hydroxylamine hydrochloride solution was mixed with an equal volume of 20 % (w/v) NaOH.

Diluted HCl. Conc. HCl diluted with an equal volume of distilled water.

$FeCl_3$ *reagent.* 10 % (w/v) Hydrated $FeCl_3$ in 0·1 N-HCl.

All the above solutions, with the exception of the acetylcholine solution, were prepared with analytical quality reagents.

The determination of cholinesterase activity

A 1 % rat-brain homogenate in phosphate saline was used as a source of specific cholinesterase; it was found to maintain its original activity for at least a week when stored in a refrigerator. Into a stoppered tube, graduated at 15 ml. and maintained at 37° in a water bath, were pipetted 2 ml. of the brain homogenate and 1 ml. phosphate saline, followed by 2 ml. acetylcholine solution. After 15 min. the reaction was stopped by the addition of 4 ml. alkaline hydroxylamine reagent. After at least 1 min., 2 ml. diluted HCl and 3 ml. $FeCl_3$ reagent were added and the contents made up to 15 ml. and the solution filtered. The solution was transferred to a 1 in. cylindrical cell and the optical density read at 540 mμ. in a Unicam DG spectrophotometer. A slight fading was noted during the first 30 min. after mixing, which was largely accounted for by a similar fading in a blank determination on 5 ml. phosphate saline submitted to the above procedure; to standardize the procedure, readings were taken 30 min. after the addition of $FeCl_3$ reagent.

A standard curve was constructed with a series of dilutions of acetylcholine; this was linear over the range of concentrations encountered in this investigation. In order to estimate the acetylcholine remaining after the enzymic hydrolysis, the measured optical density, less the blank value, was applied to the standard curve. Fig. 1 shows that the extent of hydrolysis, plotted as $\log C_o/C$, where C_o and C are the initial and final concentrations of substrate, is proportional to the time of hydrolysis until the reaction has proceeded about half-way to completion, after which the rate slows down. (All logarithms in this paper are to base 10.) This is consistent with a first-order reaction, and Fig. 2 shows the expected proportionality between $\log C_o/C$ and the concentration of enzyme when the time is kept constant at 15 min.

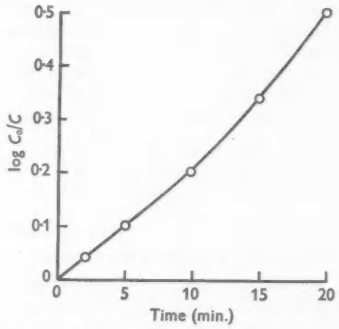

Fig. 1. Variation in extent of hydrolysis of acetylcholine ($\log C_o/C$) with time, the concentration of rat brain being 4 mg./ml.

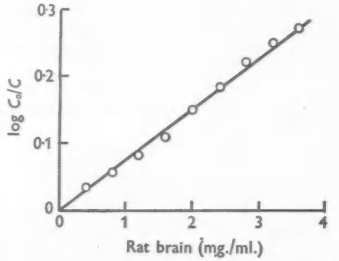

Fig. 2. Variation in the extent of hydrolysis of acetylcholine ($\log C_o/C$) with the concentration of rat brain, the period of hydrolysis being constant at 15 min.

The variation in the cholinesterase activity of different rat-brain homogenates is not large; the mean percentage hydrolysis in 15 min. from thirteen tests is 59 with a coefficient of variation of 6·4%.

Measurement of inhibition

Solutions of the inhibitors to be examined were prepared containing 100 mg./100 ml. ethanol and a series of dilutions in phosphate saline were made. To 2 ml. of the brain homogenate was added 1 ml. of the inhibitor solution and the mixture incubated at 37° for 30 min. Then 2 ml. of substrate were added and incubation continued for a further 15 min., after which the residual acetylcholine concentration C_I was estimated. If E_I is the activity of the enzyme in the presence of inhibitor, the fractional residual activity E_I/E_o is equal to $(\log C_o/C_I)/(\log C_o/C)$.

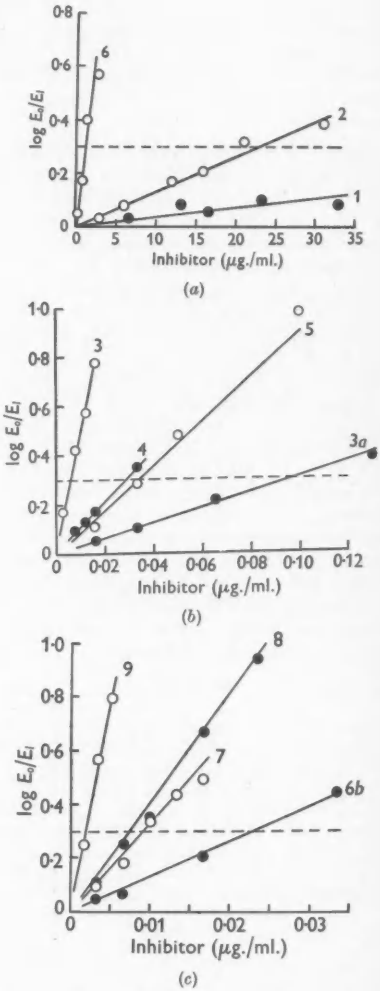

Fig. 3*a*, *b*, *c*. Variation in the term $\log E_o/E_I$ with the concentration of inhibitor; the key to the numbers referring to the various inhibitors is given in Table 1.

RESULTS

Aldridge's (1950) investigation into the kinetics of red-cell cholinesterase inhibition by parathion has indicated that it has the characteristics of a

Table 1. *Inhibition of brain cholinesterase by various preparations of parathion and related compounds*

(The inhibitory activity is the reciprocal of the concentration needed for 50% inhibition of the enzyme.)

Ref. no.	Description	Concn. for 50% inhibition (mg./ml.)	Inhibitory activity	*S*-ethyl isomer (%)
1	Parathion purified by chromatography	—	—	<0·4
2	'Pure' parathion	23	0·43	0·5
3	'Pure' parathion	0·06	17·5	9·6
3*a*	Sample 3 extracted with N-NaOH	0·96	1·04	4·0
4	Technical parathion (sample *X*)	0·28	3·6	2·0
5	Technical parathion (sample *Y*)	0·35	2·9	0·5
6	Technical parathion (sample *Z*)	1·2	0·83	0·8
6*b*	Sample 6 heated 3 hr. at 150°	0·023	43·5	16·0
7	'*OS*-Diethyl *O*-*p*-nitrophenyl thiophosphate'	0·009	111	62·0
8	'*OO*-Diethyl *S*-*p*-nitrophenyl thiophosphate'	0·007	143	0·6
9	'*OO*-Diethyl *O*-*p*-nitrophenyl phosphate' (E 600)	0·002	500	1·3

bimolecular reaction with one component in large excess. If Aldridge's equation is assumed, the inhibitor concentration should be proportional to log E_o/E_I, where E_o is the original activity of the enzyme and E_I the residual activity after inhibition. Figs. 3*a*, *b* and *c* show the results obtained when various samples of parathion and analogous compounds are investigated and the inhibitor concentration plotted against log E_o/E_I. A key to the numbering in these figures is given in Table 1. The points fall tolerably well on straight lines, which differ very widely in slope. The points at which the ordinate log 2 intersects these lines corresponds with the concentrations required for 50% inhibition of the enzyme; these values are given in Table 1. The reciprocals of the 50% inhibition concentrations are taken as a measure of the inhibitory activity of the samples.

DISCUSSION

All the samples examined for *in vitro* cholinesterase inhibition were subjected to the analytical procedure which has been described elsewhere (Gage, 1951). It became evident that, with a few exceptions, the very wide range in inhibitory activity, which is disclosed in Fig. 3 and Table 1, was related to the variation in the percentage of *OS*-diethyl *O*-*p*-nitrophenyl thiophosphate, the *S*-ethyl isomer of parathion (II). A sample of parathion which had been subjected to a repeated chromatographic purification procedure and which contained only a trace of the isomer (sample 1) had a very low *in vitro* activity; 50% inhibition could not be achieved with a saturated aqueous solution. Fig. 4 shows that the points obtained by plotting the inhibitory activity of the parathion samples against their percentage of the *S*-ethyl isomer fall approximately on a straight line passing through the origin. Samples stated to be the *S*-phenyl isomer of parathion (III) and the oxygen analogue of parathion, E 600 (IV), do not conform; these have an *in vitro* inhibition which is not attributable to their content of the *S*-ethyl isomer.

The conclusion to be drawn from this investigation is that pure parathion has no appreciable *in vitro* inhibition of cholinesterase when examined by the method described. The substrate concentration used in this investigation was $1{\cdot}2 \times 10^{-4}$M; this is considerably lower than that used by previous investigators in this field, but the very low activity of the pure sample has been confirmed with 10^{-2}M-acetylcholine by an electrometric method. It may be

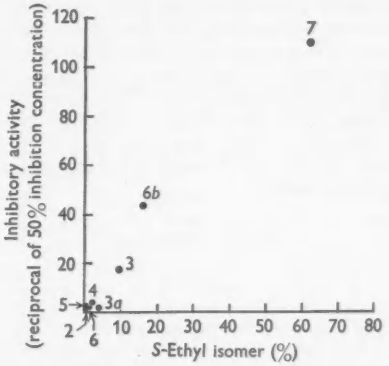

Fig. 4. Graph showing the linearity between the percentage of *S*-ethyl isomer and the inhibitory activity of the various samples. The samples are numbered as in Table 1.

concluded that the high cholinesterase inhibition ascribed by the earlier workers to parathion was due to active impurities in the samples used.

SUMMARY

1. The *in vitro* cholinesterase inhibition of a series of samples of parathion (*OO*-diethyl *O*-*p*-nitrophenyl thiophosphate) and of similar compounds has been studied using a rat-brain homogenate as a source of specific cholinesterase.

2. It has been shown that the *S*-ethyl and *S*-phenyl isomers of parathion and the oxygen analogue of parathion have a powerful inhibitory action.

3. The variable inhibition produced by samples of parathion can be entirely ascribed to contamination with the *S*-ethyl isomer of parathion, and a highly purified sample, containing only a trace of this isomer, has a very low inhibitory action.

We are indebted to Messrs Albright and Wilson Ltd., and to Messrs Plant Protection Ltd., for supplying samples of parathion and analogous materials.

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Iron Metabolism and Haemoglobin Formation in the Embryonated Hen Egg

2. SOME OBSERVATIONS ON THE EMBRYONIC BLOOD SUPPLY

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(*Received* 4 *January* 1951)

In a recent paper (Ramsay, 1950) some evidence was presented suggesting that the membranes of the developing egg contain a substantial proportion of the total quantity of haem iron in the egg, and hence of the haemoglobin and blood. This paper discusses in greater detail the changes which take place in the amount of haem iron and its distribution between the body of the embryo and its membranes during incubation.

EXPERIMENTAL

General

Eggs from Brown Leghorn hens weighing 50–60 g. were incubated for various periods and frozen as soon as possible after removal from the incubator in thoroughly prechilled acetone-CO_2. The shell was removed, and the embryo body dissected from the remainder of the egg contents. In this way the separation was easily made without loss of blood, and it is believed that the freezing was sufficiently rapid to prevent any serious redistribution of blood taking place.

Homogenates prepared with the addition of suitable quantities of ice-cold water from the embryo and the 'rest' (the 'rest' comprised yolk, white, allantoic and amniotic fluids, and the membranes, but not the shell) were separately analysed for non-haem and total Fe by the methods described below. In the interpretation of the results it has been assumed that the haem Fe in the 'rest' was derived entirely from the haemoglobin of the blood circulating in the membranes. These constitute the only living tissue outside the body of the embryo, and they are such highly vascular structures that the cytochrome and other haem compounds of the membrane cells themselves must surely be even less important quantitatively than in less vascular systems. Where necessary, the rather less justifiable assumption has been made that in the embryo body also the amount of non-haemoglobin haem Fe might be neglected. This is based on the work of Drabkin (1948), who has shown that in the bodies of several species cytochrome and myoglobin do not account for more than about 10% of the total haem.

Non-haem iron

Reagents. Dipyridyl reagent: a solution containing 2·5 M-acetate, pH 4·6, 2:2′-dipyridyl, 0·2%, $NH_2OH.HCl$, 0·5%. Ethanol, redistilled.

Procedure. Homogenate (1 ml.) was treated with 4 ml. dipyridyl reagent and the mixture heated for 5 min. in a boiling-water bath. After cooling, the contents of the tube were made up to 12 ml. with ethanol and filtered through a no. 42 paper when the precipitate had coagulated. The Unicam D.G. Spectrophotometer at a wavelength setting of 520 mμ. was found very suitable for measuring the density of the pink colour. 'Blanks' were done in the same way, but with acetate buffer alone instead of the complete reagent.

Total iron

The technique for estimation of total Fe was based on the belief that the procedure just described does in fact determine all non-haem Fe, and on the knowledge (Peters, 1947) that haem pigments can be decomposed by H_2O_2. It has been found that the decomposition is not always effective at low temperatures, perhaps because catalatic destruction of H_2O_2 interferes with the desired reaction. Moreover, H_2O_2 interferes with the subsequent dipyridyl reaction, but excess can be easily destroyed with Na_2SO_3.

Reagents. H_2O_2, 3% (w/v) in 2 M-acetate, pH 4·6.

$Na_2SO_3.7H_2O$, 40% (w/v). Neither of these reagents should be more than a week old.

Dipyridyl reagent: this was exactly twice as concentrated, with respect to all the solutes, as the non-haem Fe reagent.

Ethanol, redistilled.

Procedure. Homogenate (1 ml.) was warmed to a temperature not lower than 50° and H_2O_2 (0·2 ml.) added. The mixture was shaken occasionally during 5 min., and again after the addition of 1 ml. Na_2SO_3. After a further 5 min. dipyridyl reagent (2 ml.) was added, and the tube heated for 15 min. in a boiling-water bath. After cooling, the volume was made up to 12 ml. with ethanol and the estimation completed in the same way as for non-haem Fe.

The NH_2OH in the dipyridyl reagent is not strictly necessary in this procedure, as sufficient sulphite is always left after the destruction of excess peroxide to reduce all the Fe. It was added in order that as many features as possible might be common to the total and non-haem Fe methods. It cannot be replaced by sulphite in the latter technique, since the heating of haemin or haemoglobin solutions with sulphite and dipyridyl in weakly acid solution results in the rapid formation of the ferrous dipyridyl complex ion.

Both methods gave satisfactory recoveries of iron added to biological materials such as milk, egg yolk, or chick embryo homogenized at different stages of development. With the total Fe method the extra iron was added as oxyhaemoglobin. Possible inhibitory effects were investigated by going through the long list of substances tested by Jones (1948). Iron added as ferro- or ferri-cyanide did not react completely at pH 4·6, but these complexes are unlikely to be found in biological material. The curious apparent inhibitory effect of pyruvate observed by Jones was not found when sufficient NH_2OH was added to leave an excess after the formation of the oxime of pyruvic acid. Oxalate in the large amount of 200 mg. per tube interfered seriously with colour development, but it is considered very unlikely that in actual practice inhibitory concentrations of oxalate would be encountered.

It is well known that unincubated eggs contain no haem iron (Hill, 1931; Ramsay, 1950). In five such eggs analysed by these two methods the non-haem Fe ranged from 99 to 100·5% of the total Fe. In the embryos and 'rests' of partially incubated eggs total Fe values found by this technique agreed closely with those found by either the dipyridyl reaction or titanium titration after wet ashing, showing the absence from these materials of Fe complexes not decomposed by heating with dipyridyl and a reducing agent after preliminary treatment with H_2O_2.

Homogenates prepared from the embryos and 'rests' of eggs incubated for 14 days (in these the 'rest' contains substantial quantities of haem Fe) were heated for periods up to 2 hr. with the non-haem Fe reagent. It was found that the pink colour developed rapidly for about 3 min. and remained constant for a further 15–20 min. After this, it increased slowly in intensity until at the end of 2 hr. it was nearly as intense as that obtained after decomposition of haem pigments with H_2O_2. It was concluded that although haem pigments are slowly attacked by the non-haem Fe reagent at the boiling point, there is not, in these materials, significant decomposition in the 5 min. period adopted as a routine.

Although it was believed that the experiments described in the preceding paragraphs provided good circumstantial evidence in favour of the assumption that the difference between experimentally determined total and non-haem Fe is in fact haem Fe, it was felt that direct evidence would be preferable. Attempts were therefore made to compare the methods of this work with the pyridine haemochromogen technique of Flink & Watson (1942), which was designed for application to urine, faeces and blood plasma. The results of the comparison were disappointing, apparently because of erratic pyridine haemochromogen determinations, but they did suggest that the fundamental assumption is not entirely unsound, at least for the present experimental material.

Caution, however, is required in the application of the methods to other materials. The non-haem Fe method gives erroneous results when applied to whole blood or to any other material containing an unusually large excess of haem over non-haem Fe. This is due partly to very high and variable 'blanks' caused by solution of haem pigment in the aqueous acid ethanol medium, and partly to a significant decomposition of haem pigment in even the shortest possible heating period. Moreover, in the determination of total Fe in whole blood or liver, the decomposition of haem pigments by the amount of H_2O_2 recommended here only proceeds quantitatively if the specimen has been previously heated to 100°. Preliminary experiments indicate that the non-haem iron method is perhaps exceptionally well suited to the determination of plasma Fe.

When small quantities of Fe are being determined, the care of glassware is very important. Glass easily becomes contaminated with iron which is not, in the author's opinion, satisfactorily removed by the usual $K_2Cr_2O_7$-H_2SO_4 mixtures. In this laboratory glass is first scrubbed with detergent and then, immediately before use, rinsed with hot HCl or HNO_3 and freed from acid with distilled water only.

RESULTS

The analytical techniques have been applied to seventy-four eggs incubated for periods varying between 4 and 20–21 days. The results are shown in Figs. 1–4, where the wet weight of the embryos is plotted against haem iron in the embryo (Fig. 1), haem iron in the membranes (Fig. 2), haem iron in the whole egg (Fig. 3) and non-haem iron in the embryo (Fig. 4). A double logarithmic scale was chosen for convenience in presenting the results, since the embryo weights vary over the very wide range of 0·15–34 g. This method of plotting, moreover, may allow the detection of certain general trends which are very much less obvious when ordinary arithmetical scales are used.

Fig. 1 shows that the haem iron in the embryo body increases steadily from about 1·2 μg. at embryo weight 0·15 g. to 10 μg. at embryo weight 1 g., 120 μg. at 10 g. and 300 μg. at 20 g. At the point of hatching (embryo weight 30 g. or over) most embryos probably contain between 600 and 750 μg. haem iron. Over the greater part of the incubation period there is a fairly strict proportionality between the two sets of logarithms.

Fig. 2 shows, as might be expected, a very different state of affairs with regard to the membranes. The membrane haem iron increases, but less steadily than that in the embryo body, until it reaches a maximum of 200–300 μg. at embryo weight 16–19 g. After this it begins to decrease

extremely rapidly, until by the time the embryo weighs 30 g., the haem iron in its membranes has dropped to 60 μg., no more than it is at the end of the first week of incubation.

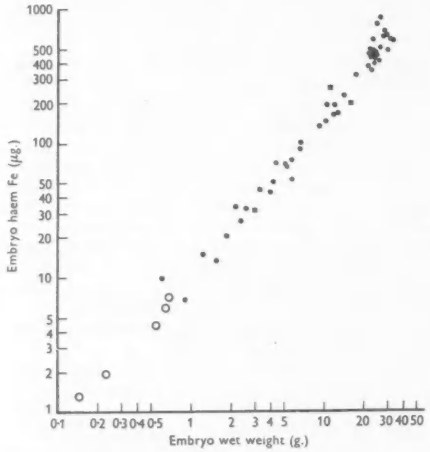

Fig. 1. Embryo haem iron during embryo growth. Note the use of logarithmic scales. ●, points from the analysis of individual embryos; ○, points from the analysis of three to six pooled embryos.

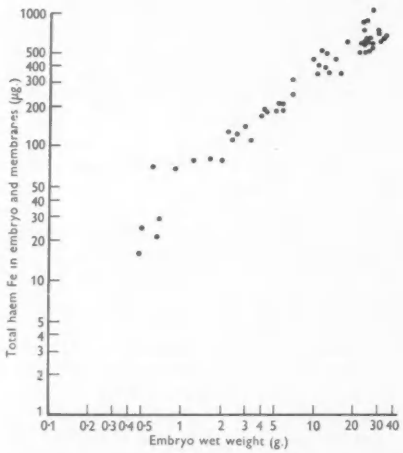

Fig. 3. Total (embryo + membranes) haem iron during embryo growth. Note the use of logarithmic scales.

When these two sets of figures are combined, as in Fig. 3, to show the increase of total haem iron with embryo weight, it can be seen that the proportionality between the two sets of logarithms is broadly maintained, at least until the sharp decrease in membrane haem iron becomes apparent towards the end of incubation.

Fig. 4 shows the increase in non-haem iron in the embryo body plotted against embryo weight. It will be observed that there is a broad proportionality between the logarithms of the two quantities

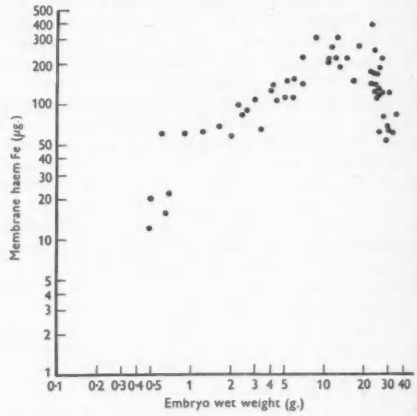

Fig. 2. Membrane haem iron during embryo growth. Note the use of logarithmic scales, and that embryo weight, not membrane weight, is the standard of comparison.

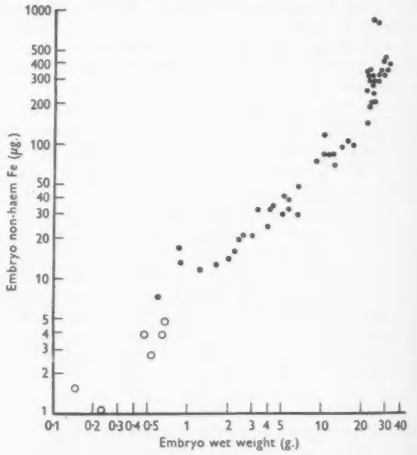

Fig. 4. Non-haem iron in the embryo during growth. Note the use of logarithmic scales. ●, points from the analysis of individual embryos; ○, points from the analysis of three to six pooled embryos.

during the greater part of incubation, but that during the last few days, after the embryo weight exceeds 20 g., there is a sharp increase in the rate of accumulation of non-haem iron. Before this stage,

the embryo contains approximately 7 μg./g. non-haem iron, while by the time the weight has reached 25 g. the figure is about 10 μg./g., and this increases even more by the time the chick is ready to hatch.

It has already been noted that haem iron in the 'rest' is assumed to be derived from the haemoglobin of the blood circulating in the membranes. Although the 'rest' in these preparations included the amnion, this membrane is so much smaller and less vascular than the others that the haem iron in the 'rest' may be safely regarded as wholly derived from the circulation in the yolk sac and the chorio-allantois. The relative importance of the two latter membranes in this respect has been the subject of eight experiments on eggs between the 10th and 16th days of incubation, a period during which the membrane circulations appear to reach their zenith. In these experiments the frozen egg-contents were separated into three parts: the embryo, the yolk with its adherent yolk sac, and the remaining parts—residual white, fluids and chorioallantois. Analyses done on these fractions suggest that at this period of development the circulation of the chorioallantois usually carries nearly as much blood as that of the yolk sac. In six experiments the chorioallantois contained 38–49 % of the blood in the membranes, but in the remaining two the values were only 23 and 25 %. In these eggs, however, the volume of the allantoic fluid (and therefore presumably the size of the containing membrane) was very much less than in the others.

The relation of embryo wet weight to incubation period was much the same in these experiments as in the work of Byerly (1932), except that in this laboratory the weight at hatching has been lower by perhaps as much as 10 %.

DISCUSSION

It is perhaps noteworthy that the average total iron content of these eggs (1·23 mg./egg in 77 eggs; S.D. ± 0·2) was significantly higher than that of the earlier series (Ramsay, 1950; 1·07 mg./egg in 115 eggs; S.D. ± 0·2). Inquiry showed that the diet of the flock had been altered in the interval, but unfortunately in several different respects. With the increased iron content of the new series of eggs, the effect of quantity of iron available on quantity of haem synthesized was much less pronounced than before.

It would appear from the results which McFarlane & Milne (1934) quoted that the total iron in the present series of eggs was probably much the same as in those used by the American authors. They separated the embryo from the rest of the egg after snipping the yolk stalk and its attendant vessels as close to the embryo body as possible, and determined the total iron in the embryo only by wet ashing and thiocyanate colorimetry or titanium titration. Their results agree fairly well with those obtained in the comparable portions of the present work.

Since the circulations of the yolk sac and the chorioallantois are the channels through which the embryo breathes, excretes, and is fed, one might expect that they would increase in magnitude throughout the course of incubation. In point of fact this does not seem to be the case, as may be seen from Fig. 2, or in more striking terms from Fig. 5,

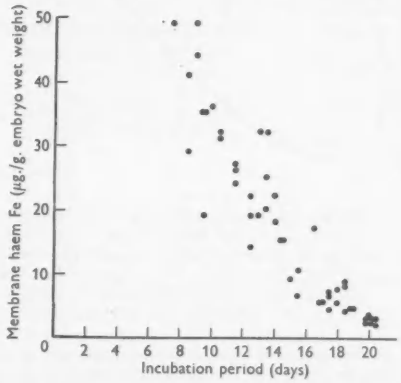

Fig. 5. Membrane haem, as quantity per unit wet weight of embryo (μg./g.), during incubation of hen eggs.

which shows a steady decrease in the quantity of membrane haem per unit weight of embryo body between the 9th and 21st days of incubation. Such figures cannot be related to blood volume changes unless the trend of blood haemoglobin concentration figures is also known. Haemoglobin concentrations in chick embryo blood have not yet been undertaken in this laboratory, but a series of analyses was published by Zorn & Dalton (1937). Calculations made with the aid of their figures suggest that the total membrane blood volume may be about 0·3 ml. in 9 days, 0·45–0·5 ml. at 13–17 days, and perhaps less than 0·2 ml. at 20 days. It appears likely that at 9 days, when the embryo is growing at the rate of about 0·15 g. dry weight per day, there is more blood in the membranes than towards the end of incubation, when the absolute rate of growth may be four or five times greater. A somewhat similar state of affairs was encountered by Barcroft and his collaborators in the investigation of foetal physiology in the sheep (Barcroft, 1946). In that species the proportion of the total blood volume which is present in the placenta decreases from 50 % between 90 and 100 days to about 15 % at 140 days, 1 week before parturition. The absolute volume of

blood in the placenta changes but little during the same period, in spite of the growth of the foetus. Barcroft and his colleagues demonstrated experimentally the truth of the most probable explanation, that the lack of increase in placental blood volume is compensated by a substantial rise in arterio-venous pressure difference and a concomitant rise in the rate of circulation. It would be interesting to know whether the chick embryo deals with its related problem in the same way. Fig. 6 illustrates the general similarity between sheep and fowl in the matter of blood distribution between the embryo and its physiological appendages. In both cases about two-thirds of the development time elapses before the embryo body contains half of the total embryonic blood.

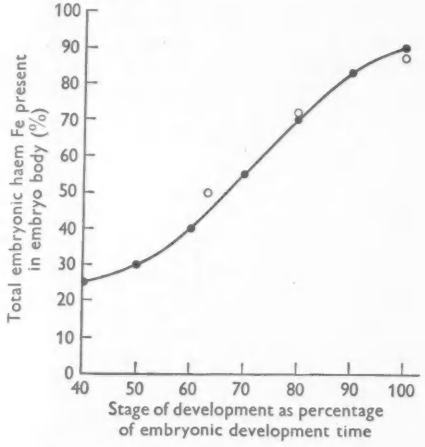

Fig. 6. Percentage of total embryonic haem iron or blood present in the embryo body at different stages of development. Stage of development expressed as percentage of total incubation or gestation period. (100% = 21 days for hen, 147 days for sheep.) ●——●, points for the hen, calculated from this work; ○, points for the sheep, calculated from results quoted by Barcroft (1946).

Inspection of Fig. 2 poses the question of the fate of the haemoglobin which leaves the membrane circulations during the last few days of incubation. The absence of a sharp upward turn in Fig. 1 at the point corresponding to that at which the membrane haem begins to decrease shows that the rate of increase in the embryo body is insufficient to explain the disappearance of the membrane haem by a process of transference into the embryo body, unless it is assumed that the haemopoietic process is conveniently interrupted for a few days. Such an occurrence would seem highly improbable, and two further facts point towards a rather different course of events: the late embryo or newly hatched chick has a gall-bladder distended with highly pigmented bile, and during the last few days of incubation, as may be seen from Fig. 4, the rate of accumulation of non-haem iron in the embryo body shows a sharp upward trend. In this respect the non-haem iron behaves differently not only from haem iron, but also from such diverse compounds as choline, creatine, methionine and the nucleic acids (G. S. Boyd, T. M. Sutherland, private communications). It seems highly probable, therefore, that towards the end of incubation haemoglobin degradation is either initiated or accelerated in such a way that the 'slack' created by the disappearance of the membranes is neatly taken up. The iron liberated is retained in the embryo body, where it presumably makes a substantial addition to the stored iron of the young chick.

SUMMARY

1. Total and non-haem iron have been determined in the embryo and the remaining portions of fertile eggs frozen and dissected after incubation for various periods.

2. Haem iron in the embryo body increases in such a way that the logarithm of the amount present is directly proportional to the logarithm of the wet weight. This relationship holds until the last 2 or 3 days of incubation. The quantity present is about 10 μg. at embryo weight 1 g., 300 μg. at 20 g., and 600–750 μg. at 30–35 g.

3. Haem iron in the membranes increases to a maximum of 200–300 μg. at embryo weight 16–19 g., and then decreases sharply. While the embryo increases in weight from 3 to 18 g., the proportion of the membrane haem in the chorioallantois remains about 40%. The proportion of the total haem which is present in all the membranes decreases from 75% at embryo weight 2 g. to 50% at 14 g. and 10% at 30–35 g. A comparison is made between these figures and the state of affairs in the uterus of the pregnant sheep, as described by Barcroft (1946).

4. Evidence is presented that towards the end of incubation a quantity of haem is destroyed which is broadly equivalent to that disappearing from the membranes. The liberated iron accumulates in the embryo, presumably as a store.

The author is grateful to the staff of the Poultry Research Centre for their help in supplying eggs, to Mr D. Temple for technical assistance, and to the Agricultural Research Council for a grant from which the expenses of the work were defrayed.

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Studies in Vitamin A

15. THE VARIATIONS IN THE SERUM β-CAROTENE AND VITAMIN A LEVELS OF COWS NEAR PARTURITION

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(*Received* 10 *January* 1951)

Kuhlmann & Gallup (1944), Braun (1945*a*) and Sutton, Kaeser & Soldner (1945), working in the United States, have reported that at or immediately after parturition the serum carotenoid and vitamin A levels of cows drop sharply, to recover slowly during the next 14–21 days. It was considered important to find out whether such a situation obtained with cows kept under normal English farming conditions, and to this end the work reported here was carried out during 1948.

EXPERIMENTAL

Animals. The cows used in this investigation belonged to the Veterinary Science Division of the Research Department of Boots Pure Drug Co. They formed a small mixed herd used for a long-term experiment on the spread of *Streptococcus agalactiae* infection, but this experiment cannot be considered to affect in any way the vitamin A and carotenoid metabolism of the cows. The general details of the experimental animals are given in Table 1. During the period of the investigation (May–November, 1948) all the cows except no. 7 were out at grass day and night until 17 October, having been turned out on 11 April. Cow no. 7 was kept in after early October because of an illness; after 17 October cows nos. 2, 3 and 7–9 were out at grass only during the day (approx. 5 hr.) and received a daily supplement of third-year seeds hay, up to 20 lb. (9·1 kg.) of kale and concentrates according to milk yield. Cows nos. 4–6 remained out day and night until the investigation ended. On calving the cows received a supplement of 8 lb. (3·6 kg.) per day of a production ration consisting of 1 part bran, 1 part oats, 1 part flaked maize (Kosistos) and 1 part dairy nuts. Throughout the period under investigation there was always plenty of grass available, for, owing to the wet summer, the pasture never dried up.

Determination of serum β-carotene and vitamin A. Blood samples were drawn from the jugular vein into McCartney bottles and immediately posted to Liverpool, where they arrived in good condition, showing little or no haemolysis. The serum was separated from the clot by centrifugation and stored at 0° until it was assayed. Determinations were always carried out as soon as possible after arrival and never more than 48 hr. later. It was assumed that no loss of carotenoids and vitamin A had occurred in transit. The carotenoids and vitamin A were extracted from the serum by the method in general use in this laboratory (Glover, Goodwin & Morton, 1947*a*). Owing to the very high concentration of β-carotene in the extracts, direct determinations of vitamin A were impracticable, because any correction applied

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Table 1. *Details of the cows used during the present investigation*

Cow no.	Date of birth	Breed	No. of days dry before calving	Date of calving
1	Jan. 1944	Ayrshire ×	57	30 Apr. 1948
2	Jan. 1944	Ayrshire ×	113	30 May 1948
3	Dec. 1944	Ayrshire	1st calf	6 Sept. 1948
4	Mar. 1945	Ayrshire	1st calf	8 Sept. 1948
5	Dec. 1944	Ayrshire	1st calf	21 Sept. 1948
6	Jan. 1945	Shorthorn (Pedigree)	1st calf	15 Nov. 1948
7	Jan. 1945	Ayrshire	1st calf	21 Nov. 1948
8	Feb. 1944	Ayrshire (Pedigree)	1st calf	Feb. 1948
9	Oct. 1944	Ayrshire	1st calf	Feb. 1948

for the presence of β-carotene would have been much too great; consequently the extracts were chromatographed on defatted bone meal (Mann, 1943; Goodwin & Morton, 1946; Glover, Goodwin & Morton, 1947*b*) before determinations were made. The blood extract, dissolved in a little light petroleum (b.p. 40–60°), was poured on to a bone-meal column; elution with light petroleum removed the β-carotene together with any small amounts of vitamin A esters which were present. The xanthophylls and free vitamin A which remained in the column were then eluted with acetone.

The light petroleum fraction was made up to a convenient volume and the amount of β-carotene present determined by measuring the E 450 mμ. value of the solution in a Beckman spectrophotometer. The vitamin A esters present in this fraction were ignored because they form only a minor fraction of the total serum vitamin A (Glover *et al.* 1947*a*). The fraction containing the xanthophylls (lutein is by far the major pigment present) and vitamin A was then examined for vitamin A. No attempt was made to separate the lutein from the vitamin A because, being only a minor component of the total serum carotenoids (about 5%), it did not interfere seriously with the $SbCl_3$ test for vitamin A, and this slight interference could be allowed for by applying a correction, which never amounted to more than 5% of the uncorrected value. The correction applied was based on our observation that the ratio $E^{1\%}_{1\,cm.}$ 446 mμ./$E^{1\%}_{1\,cm.}$ 617 mμ. is 6·35 for a chromatographically homogeneous sample of lutein isolated from grass.

The xanthophyll-vitamin A fraction was made up in a known volume of acetone, the E 446 mμ. value measured in the Beckman instrument, and the $E^{1\%}_{1\,cm.}$ for the serum calculated in the usual way. The acetone was then removed under a stream of N_2, the residue dissolved in 1 ml. of $CHCl_3$ and the $SbCl_3$ test carried out at 617 mμ. again using the Beckman instrument (Glover *et al.* 1947*a*; Goodwin & Gregory, 1948). The observed $E^{1\%}_{1\,cm.}$ 617 mμ. value was then corrected by subtracting from it the $E^{1\%}_{1\,cm.}$ 446 mμ. value divided by 6·35.

RESULTS

Changes in the serum β-carotene and vitamin A levels around parturition. The variations in the concentrations of both these constituents were followed in six cows (nos. 2–7) and all showed a gradual decline during the last week of pregnancy until at, or just after, parturition they were, on the average, between 60 and 70% of their normal values. The levels of all the cows except no. 7 then gradually improved until at 1 month post-partum they were approaching the normal levels. Following parturition, cow no. 7 behaved quite abnormally, for neither the β-carotene nor the vitamin A level recovered from the drop at parturition, but continued to fall until the end of the experiment. No obvious clinical condition could account for this at the time (end of November), but the animal had been ill early in October.

The results obtained for the serum vitamin A and β-carotene values for cows nos. 2–6 around parturition are recorded in Figs. 1 and 2 as scatter diagrams. The basic value for each cow (except

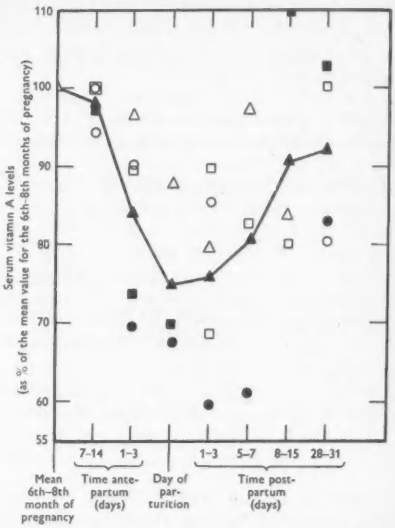

Fig. 1. The variations in the serum vitamin A levels of cows near parturition. The mean values for the 6th, 7th and 8th months of pregnancy are taken as 100 and the levels around parturition adjusted accordingly. ○, cow no. 2; ●, cow no 3; □, cow no. 4; ■, cow no. 5; △, cow no. 6; ▲——▲, mean values.

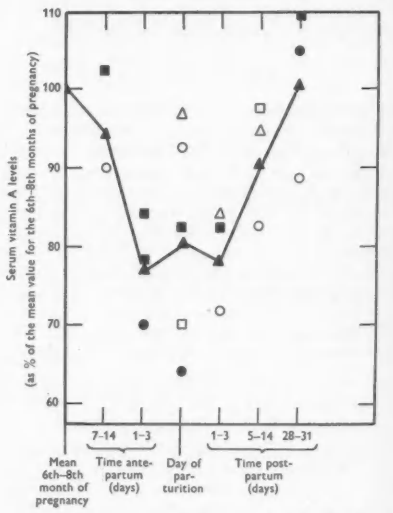

Fig. 2. The variations in the serum β-carotene levels of cows near parturition. The mean values for the 6th, 7th and 8th months of pregnancy are taken as 100 and the levels around parturition adjusted accordingly. ○, cow no. 2; ●, cow no. 3; □, cow no. 4; ■, cow no. 5; △, cow no. 6; ▲——▲, mean values.

no. 2) has been taken as the means of the routine monthly values obtained during the 6th, 7th and 8th months of pregnancy and has been given the value 100. The values near parturition were then adjusted accordingly. The values obtained 14 days before calving for cow no. 2 were taken as her base line for no previous values were available; this is justified because reference to the diagrams will show that no significant drop had occurred in the remaining cows when examined between 7 and 14 days ante-partum. The first serum value obtained for cow no. 1 was for the day of parturition, but the values obtained post-partum all showed an increase on these values. Cows nos. 8 and 9 were not followed through parturition.

Seasonal changes in serum β-carotene and vitamin A levels. The monthly serum levels for vitamin A and β-carotene obtained between May and October 1948 are recorded in Tables 2 and 3. An analysis of variance of these results revealed no significant month to month variations for either component.

The vitamin A and β-carotene content of colostrum. The values obtained on a number of samples of colostrum from cows nos. 1, 2 and 9 are recorded in Table 4. Very high values were obtained for early colostrum, but these fell off rapidly until 14 days after parturition they were generally less than 10% of their original values. This confirms previous observations (see e.g. Sutton, Warner & Kaeser, 1947; Ganguly, Kon & Thompson, 1947). Colostrum obtained 2 and 6 days before delivery was as high as that obtained on the day of calving. Samples of colostrum from cow no. 8 were examined and the results calculated per ml. of colostrum (the lipid content was not measured); in this case the values of vitamin A and β-carotene fell from 1·73 to 2·44 μg./ml., respectively, at parturition to 0·41 and 0·75 μg./ml. 7 days later.

Table 2. *The serum vitamin A levels* (μg./100 ml.) *of cows during May–October* 1948

	Month					
Cow no.	May	June	July	Aug.	Sept.	Oct.
2	Calved	22	27	31	30	29
3	33	32	43	34	Calved	29
4	46	38	34	34	Calved	35
5	33	29	20*	28	Calved	31
6	30	32	33	29	30	30
7	30	46	38	43	38	—
8	33	31	31	35	30	29
9	34	31	30	36	—	30

* Doubtful value.

Table 3. *Serum β-carotene values* (μg./ml.) *of cows during May–October* 1948

	Month					
Cow no.	May	June	July	Aug.	Sept.	Oct.
1	Calved	8·9	6·5	9·9	9·6	10·2
2	Calved	15·3	16·8	19·0	16·4	12·6
3	18·2	14·0	13·6	—	Calved	14·4
4	9·1	10·6	8·6	8·6	Calved	11·2
5	8·8	8·2	9·9	9·8	Calved	7·3*
6	6·3	5·1	3·9	4·0	5·4	5·4
8	30·0	28·0	—	22·0	15·0	16·0
9	12·0	11·0	11·2	12·2	13·0	—

* Only 5 hr./day on grass.

Table 4. *The vitamin A and β-carotene content of colostral fat*

(All values expressed as μg./g.)

	Time pre-partum (days)				Day of parturition		Time post-partum (days)					
	6		2				1–2		7		14	
Cow no.	Vit. A	β-Carotene	Vit. A	β-Carotene	Vit. A	β-Carotene	Vit. A	β-Carotene	Vit. A	β-Carotene	Vit. A	β-Carotene
1	—	—	—	—	31	98	12	13	1·8	5·5	—	—
2	—	—	—	—	55	139	37	62	4·0	10·1	—	—
9	37	112	35	131	—	—	15	37	—	—	1·2	9·5

DISCUSSION

In considering the significance of the fall in the serum β-carotene and vitamin A levels at parturition, it must be taken into account that the serum vitamin A is functional whilst the serum β-carotene is, in all probability, of no physiological value, merely being that which has escaped conversion into vitamin A in its passage across the intestinal wall. When the same variations are noted for two blood components, one physiologically active, the other inert, it is reasonable to assume that the changes have no significance for either, but merely indicate a general variation in the concentration of blood constituents at parturition. This conclusion is supported by the recent observations that similar changes associated with parturition of cows have been reported for plasma lipids (Niedermeiler, Smith & Whitehair, 1949) and for calcium and phosphorus (Niedermeiler *et al.* 1949; Wilson, 1951) and vitamin E (Parrish, 1949). (There is, however, one report (Kaay, Teunissen, Emmerie & Eekelen, 1949) which states that plasma vitamin E levels do not alter at parturition.)

During the 6 months covered by this investigation there were no statistically significant variations in the serum vitamin A or β-carotene levels except at parturition. Because of the 'buffering' action of the liver vitamin A (Glover *et al.* 1947*a*), there is no reason to expect major changes in the vitamin A levels, but changes in the β-carotene levels might have been expected, for it has been noted on many occasions that there is a sudden increase in the β-carotene levels during the 'spring flush' (in late March and April in Britain). These high levels are maintained until about midsummer when usually a fall is observed owing to the drying up of the pasture during the hot months. The values then increase to a second (lower) maximum in early autumn, when a second crop of pasture appears (e.g. Lord, 1945). In the case of animals in the present experiment, the cows had already been out to grass a month before the first blood samples were taken and thus the spring rise had already occurred. Further, the summer of 1948 had rainfall above the average and during May–November there was always a good 'bite' of grass. At no period did the pasture dry up.

The significance of the serum vitamin A/β-carotene (A/C) ratio in cows. Braun (1945*a*, *b*) first attempted to attribute significance to the *A/C* ratio in cattle. He found the ratio to decrease with increasing carotene concentration until the latter reached about 9 μg./ml.; above this level the ratio remained constant. This was taken to indicate a decreasing efficiency of conversion of β-carotene into vitamin A with increasing β-carotene intake, until, at and above the level quoted, a constant proportion is converted.

The *A/C* ratios have been calculated for the results recorded in Tables 2 and 3 and analysed statistically. The regression lines connecting β-carotene levels with the *A/C* ratio indicate a highly significant correlation between these two parameters over the whole range of β-carotene levels examined. Similar analyses indicate no significant correlations either between the *A/C* ratio and the vitamin A levels or between the vitamin A and β-carotene levels themselves. This statistical demonstration, that the *A/C* ratio is solely dependent on the β-carotene levels of the blood, confirms the experimental observations that serum vitamin A levels are relatively stable, owing to the 'buffering' of the liver vitamin A, whilst the plasma β-carotene levels are extremely labile, quickly reflecting variations in intake. No greater significance than this can be ascribed to the *A/C* ratio for normal cattle.

SUMMARY

1. The tendency of the serum β-carotene and vitamin A levels of cows to drop at, or just after, parturition has been confirmed. These changes are considered not significant for vitamin A and β-carotene *per se*, but are probably only a reflexion of general changes in the concentration of blood constituents at parturition.

2. No seasonal variations in serum vitamin A or β-carotene levels of cows at grass occurred during May–October 1948.

3. The high values for the vitamin A and β-carotene content of early colostrum and their rapid post-partum decline has been confirmed. Colostrum drawn up to 6 days ante-partum is also very rich in vitamin A and β-carotene; 94% of the colostral vitamin A is esterified.

4. The significance in cows of the ratio serum vitamin A/β-carotene is discussed.

We wish to thank Sir Jack Drummond, F.R.S., for permission to use the herd of cows belonging to Boots Pure Drug Co. Ltd. and Mr D. L. Hughes and Mr R. Hensen for arranging the collection of blood samples. We are also indebted to Prof. R. A. Morton, F.R.S., for his interest and help during this investigation. One of us (A.A.W.) participated in this work as the holder of the Goldsmiths' Company Fellowship of the Animal Health Trust.

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The Constitution of Hexadecatrienoic Acid from the Glycerides of Rape (*Brassica napus* L.) Leaf

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Apart from hexadec-9-enoic acid, the C_{16} unsaturated acids are of somewhat rare occurrence. From sardine oil there has been isolated hexadeca-6:10:14-trienoic acid (Toyama & Tsuchiya, 1935) and a hexadecatetranoic acid (Tutiya, 1940, 1941). More recently Shorland (1945) reported the presence of hexadecatrienoic acid in the glycerides of rape leaf.

The purpose of the present work is to determine more precisely the constitution of the hexadecatrienoic acid found in rape-leaf glycerides. In addition, the method of Armstrong & Hilditch (1925), commonly used to locate the position of the double bonds by disruptive oxidation, has been examined using linolenic acid, the constitution of which has already been determined by previous workers (cf. Erdmann, Bedford & Raspe, 1909). This step has been deemed advisable in view of difficulties which have occasionally been experienced by other workers in the isolation of the oxidation products from polyethenoid acids (cf. Shinowara & Brown, 1940).

EXPERIMENTAL

Oxidation of ethyl linolenate

Ethyl linolenate used for this work was prepared as described by McCutcheon (1938, 1940). Spectroscopic analysis by the method of Hilditch, Morton & Riley (1945) showed the presence of 95·5% (0·2% conjugated) triene acids and 2·7% (1·7% conjugated) diene acids, while the iodine value 245·8 (theoretical for ethyl linolenate 248·9) also confirmed the purity of the preparation.

The ester (8·1 g.) was dissolved in 140 ml. of acetone, and finely divided $KMnO_4$ (75 g.) was added (cf. Armstrong & Hilditch, 1925) gradually under reflux until vigorous reaction ceased. The mixture was then boiled for a further 4 hr.

After removal of the acetone on a water bath, the residue was extracted with 600 ml. of hot 5% (w/v) KOH and the extract concentrated to 150 ml. (cf. Haworth, 1929). The extract was acidified with dil. H_2SO_4 to congo red, and the volatile acids separated by steam distillation. By comparing the amount of 0·1 N-KOH required for neutralization with the dried weight (3·41 g.) of the K salts (cf. Dolby, Nunn & Smedley-MacLean, 1940) the equivalent of the volatile acids was found to be 71·3 as compared with 74·0 required for propionic acid. To test the validity of the method a sample of propionic acid was steam distilled as above and gave an equivalent of 74·9.

After extraction of the non-volatile residue with ether, the extract was boiled with water and filtered to remove any oily insoluble matter. Recrystallization from hot water yielded 1·53 g. crystals, equiv. 93·1, m.p. 104·5–106·5°. From the mother liquors a further crop of crystals (1·42 g., equiv. 91·0) was obtained. By applying counter-current distribution methods (Craig, 1944) to 0·117 g. of this latter fraction, using a mixture of 4 : 3 by volume ether-light petroleum and 20% aqueous ethanol, there was obtained, in addition to azelaic acid, 0·016 g. of suberic acid, equiv. 87·8, m.p. 131–137° (not depressed by admixture with authentic suberic acid).

Other similar oxidation experiments with ethyl linolenate as well as with methyl oleate also indicated that in addition to azelaic acid there were produced small but variable amounts of suberic acid.

Oxalic acid was estimated as described by Haworth (1929), the yield amounting to 60% of the theoretical. Malonic acid was not detected, indicating presumably, as was found by Haworth (1929), that under these conditions this acid is further oxidized to yield oxalic acid.

Oxidation of methyl hexadecatrienoate

The acids (*a*) 33·0 g. and (*b*) 35·4 g. from the glycerides of two separate samples of rape leaf were submitted to an acetone separation (10 vol. three times at −30°), and the soluble acids (*a*) 29·6 g. and (*b*) 32·1 g. converted to methyl esters. The esters were fractionated in an electrically heated, packed 60 cm. column of the type described by Longenecker (1937), and gave the results shown in Table 1.

Table 1. *Fractionation of methyl esters of acids from rape leaf*

(*a*)

Fraction no.	Wt. (g.)	B.p./about 0·1 mm. (°)	Saponification equivalent	Iodine value
1	1·79	101–105	242·6	105·3
2	1·78	105–107	264·1	206·4
3	2·32	107–114	269·0	200·3
4	2·28	114–125	275·3	186·8
5	2·51	125–127	289·8	217·4
6	2·46	127	289·9	223·8
7	2·74	127–130	292·1	227·3
8	3·25	130	292·6	233·3
9	3·53	130	292·9	229·3
Residue	4·56	—	346·5	166·6
Total weight	27·82	—	—	—

(*b*)

Fraction no.	Wt. (g.)	B.p./about 0·2 mm. (°)	Saponification equivalent	Iodine value
1	1·35	114–118	245·2	119·5
2	1·90	118–123	264·2	187·4
3	1·62	123–125	269·8	189·5
4	1·89	125–135	278·7	194·5
5	2·30	135–139	283·6	206·2
6	3·45	139	285·5	210·3
7	2·43	139	287·4	213·0
8	3·27	139–141	289·5	213·4
9	3·21	141–142	290·2	218·5
10	1·80	142	289·0	217·2
Residue	5·86	—	370·0	157·4
Total weight	29·08	—	—	—

Fractions 2–4 from (*a*) and 2–5 from (*b*) were combined separately and crystallized from 15 vol. of methanol at −15°; the soluble portions on fractionation gave the results shown in Table 2. Fractions 2–4 from (*a*) and 2 and 3 from (*b*) (Table 2) were further separated by crystallization of the corresponding acids from 10 vol. of acetone at −78°. The bulked acetone-soluble acids (4·2 g.) had a saponification equivalent of 250·7 and an iodine value of 277·1. (Theoretical values for hexadecatrienoic acid, saponification equivalent 250·0, iodine value 304·6.) Hydrogenation of 0·18 g. of the methyl esters, as described by Reasenberg, Lieber & Smith (1939), yielded on crystallization from methanol at −15°, 0·17 g. insoluble esters, m.p. 28·5–29·5°, not depressed by admixture with genuine

Table 2. *Fractionation of acetone-soluble methyl esters*

(*a*)

Fraction no.	Wt. (g.)	B.p./about 0·1 mm. (°)	Saponification equivalent	Iodine value
1	0·16	76–102	—	216·4
2	0·56	102–107	261·1	219·0
3	0·54	107	266·2	244·4
4	1·20	107–121	264·7	243·3
Residue	1·21	—	283·9	189·1
Total weight	3·67	—	—	—

(*b*)

Fraction no.	Wt. (g.)	B.p./about 0·1 mm. (°)	Saponification equivalent	Iodine value
1	0·26	76–98	255·5	108·4
2	1·69	98–106	262·9	225·5
3	1·37	106–115	266·5	218·8
4	0·78	115–120	281·7	207·4
Residue	2·29	—	293·4	208·5
Total weight	6·39	—	—	—

methyl palmitate. Spectroscopic measurements before and after alkali isomerization (Mitchell, Kraybill & Zscheile, 1943), after allowing for the differences in molecular weight between C_{16} and the C_{18} unsaturated acids, indicated the presence of

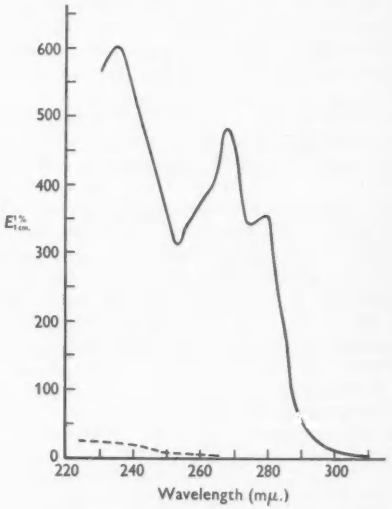

Fig. 1. Absorption spectrum of methyl hexadecatrienoate before (- - - -), and after (——) isomerization, according to the method of Mitchell, Kraybill & Zscheile (1943).

85·9% (0·2% conjugated) triene acids and 3·3% (1·8% conjugated) diene acids. The absorption curve is shown in Fig. 1.

Two separate batches (*c*) 1·86 g. and (*d*) 2·03 g. of methyl esters from the C_{16} highly unsaturated acids were oxidized and the equivalents (*c*) 70·9 and (*d*) 71·3 of the volatile acids determined as for ethyl linolenate. From the anhydrous acids (1·05 g., equivalent 71·0) prepared by distillation of the dried potassium salts from phosphoric acid saturated with P_2O_5 there was obtained using the method of Kuehn & McElvain (1931), 0·74 g. of anilide. Recrystallization of the anilide from water yielded 0·55 g. propionanilide, m.p. 104–104·5°, not depressed by admixture with authentic propionanilide.

Extraction of the non-volatile acids with ether yielded (*c*) 0·82 g. and (*d*) 0·99 g. of impure acids.

Attempts were made, without marked success, to purify (*c*) by counter-current distribution and by chromatographic adsorption from acetone solution using a column of anhydrous sodium carbonate. The latter technique, however, resulted in the separation of some impure acid, m.p. 96–98°, raised to 99·5–102° on admixture with genuine pimelic acid (m.p. 104·5–105·5°). Crystallization of 0·55 g. (*c*) from benzene followed by sublimation *in vacuo* and recrystallization from the same solvent yielded 0·42 g. acid, equivalent 79·3, m.p. 91–96·5°.

By similar procedure, but recrystallizing twice from benzene, 0·99 g. (*d*) yielded 0·62 g. acid, equivalent 79·1, m.p. 95–98°, which was raised to 100·5–103·5° when mixed with pimelic acid. The results thus indicate the presence of pimelic acid, but further attempts at more complete purification by crystallization were unsuccessful.

The amounts of oxalic acid determined, as previously described, represented (*c*) 72% and (*d*) 68%, respectively, of the yields that would be expected from a compound with three double bonds separated by two methylene groups.

DISCUSSION

The extensive work of Green & Hilditch (1937) on the oxidation of hydroxy acids with aqueous alkaline permanganate indicates that although this technique yields mainly azelaic acid in the case of the $\Delta^{9:10}$-polyethenoid acids, nevertheless, there are produced in addition by no means negligible amounts of suberic acid. For this reason they suggest that oxidation with aqueous alkaline permanganate compares unfavourably with oxidation by ozone or by potassium permanganate in acetone solution as a method for the location of the double bonds. The present results generally confirm the constitution of linolenic acid as first proposed by Erdmann *et al.* (1909) in that the expected products of oxidation, namely, propionic, oxalic and azelaic acids have been obtained. Nevertheless, the indication in the present work of substantial amounts of suberic acid in the oxidation products from ethyl linolenate as well as in the oxidation products from methyl oleate suggests that the objection raised by Green & Hilditch (1937) to the use of aqueous alkaline potassium permanganate may also apply, though to a lesser extent, when the oxidation is carried out with potassium permanganate in hot acetone solution. While admitting the possibility that the Haworth (1929) alkali extraction procedure used in this work for ethyl linolenate might have promoted the formation of suberic acid, the presence of this acid in the oxidation products of methyl oleate could not have arisen in this way, since the extraction of the oxidation products was carried out exactly as described by Armstrong & Hilditch (1925). It is therefore apparent that even when the latter method is used there is need for some caution in its use for the determination of the structure of unknown acids.

The present work confirms the earlier observations of Shorland (1945) in regard to the occurrence of a hexadecatrienoic acid in the rape-leaf glycerides. Allowing for the general nature of the oxidation of polyethylenic acids with potassium permanganate in acetone solution, the yields of propionic, pimelic and oxalic acid obtained from this hexadecatrienoic acid, together with the spectroscopic evidence before and after alkali isomerization, establish the constitution of the acid as hexadeca-7:10:13-trienoic acid, and distinct from hiragonic acid, hexadeca-6:10:14-trienoic acid, isolated by Toyama & Tsuchiya (1935) from sardine oil.

Hilditch (1948) has discussed the structural relationships of the natural unsaturated higher fatty acids and has indicated the fact that most natural straight-chain acids share a somewhat singular partial resemblance to oleic acid in that one or other part of their molecules is identical with that of one-half of the oleic acid molecule. The C_{16} unsaturated acid from rape-leaf glycerides would therefore appear to have a somewhat unusual structure, but one which nevertheless resembles linolenic acid, of which it could be precursor or from which it could be derived by β-oxidation.

In general, it has been found that the few leaf fats which have so far been examined are more or less similar in composition containing as the main constituent, linolenic acid (cf. Shorland, 1944, 1945). Although C_{16} highly unsaturated acids have been searched for in other leaf fats so far examined by one of us (Shorland, 1950) these acids have not been found, nor have they been detected in the phosphatide fraction of the rape.

SUMMARY

1. Oxidation of ethyl linolenate by potassium permanganate in acetone solution generally confirmed the structure proposed by other investigators,

but in addition to azelaic acid there was also produced some suberic acid.

2. Oxidation with potassium permanganate in acetone solution of methyl hexadecatrienoate isolated from rape-leaf glycerides yielded pimelic, oxalic and propionic acids. This evidence in association with the spectroscopic results before and after isomerization show that this acid has the formula

$$CH_3.CH_2.CH{=}CH.CH_2.CH{=}CH.CH_2.CH{=}CH(CH_2)_5.COOH.$$

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Further Observations on the Action of Chymotrypsin on Insulin

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The action of crystalline chymotrypsin on insulin has recently been described (Butler, Dodds, Phillips & Stephen, 1948) and the biological activity of the products has been investigated (Phillips, 1951). The degradation of the hormone by this enzyme gave a large fragment, the core, of molecular weight about 5000 (Butler, Phillips, Stephen & Creeth, 1950) and a mixture of small peptides which have been partially separated by paper chromatography (Phillips, 1949).

In this paper the degradation has been further studied: (*a*) to see whether the products of proteolysis cause any inhibition of the enzyme, (*b*) to locate the pH optimum for the reaction (this has been found to have two optima) and (*c*) to define the general character of the reaction, which has been found to be of the 'all-or-none' type of Tiselius & Eriksson-Quensel (1939).

EXPERIMENTAL

Insulin. Crystalline bovine insulin from Boots Pure Drug Co. was used, assaying 22–23 units/mg.

Chymotrypsin. Part of a sample of α-chymotrypsin kindly given by Dr M. Kunitz was used. The digestions were carried out in a similar way to the previous work, using approximately 3×10^{-4} T.U. (trypsin units) of enzyme/5 mg. insulin in 1 ml. of buffer at 25°. The trypsin unit referred to here is the quantity of enzyme which will liberate 1 mmol. of tyrosine from haemoglobin in 1 min. at 35·5°. According to Northrop, Kunitz & Herriott (1948) 0·038 trypsin unit is equivalent to 1 mg. of protein N.

Nitrogen. Amino N, non-protein N (N.P.N.) and total N were determined as in the previous papers. N.P.N. is expressed as % of total N.

Enzyme activity. This was determined by the method of Anson (1938).

Tyrosine. The colour given with the phenol reagent of Folin & Ciocalteu (1927) was measured, there being no tryptophan in insulin to interfere with it.

Insulin activity. This was determined by measuring the blood sugar levels (Folin & Wu, 1929) immediately prior to and 1, 2 and 3 hr. after subcutaneous injection into 1·5–2·5 kg. rabbits which had been starved 18 hr., and which had recently been shown to give a 40–60% maximum fall when given 1 unit of insulin.

RESULTS

The effect of the products of digestion of insulin by chymotrypsin on the activity of the enzyme

This effect has been estimated in three ways: by the remaining enzyme activity in the digest when tested against a standard substrate (haemoglobin); by digestion of insulin in the presence of urea; and

by measuring the digestion of insulin in the presence of various amounts of whole digest and the separated products of digestion.

There is a spontaneous loss of activity in chymotrypsin solutions which are allowed to stand at 25°. For example, a solution containing $7{\cdot}8 \times 10^{-4}$ T.U./ml. in phosphate buffer, pH 8·3, lost 20–30% of its activity in 20 hr., although a 50% decrease required 90 hr. Measurements in the presence of insulin were lacking in precision, since the medium being assayed already contained tyrosine-bearing peptides when added to the standard blood substrate. As these peptides were soluble in trichloroacetic acid (TCA), a high 'blank' value resulted. In spite of this it has been possible to show that there is residual chymotryptic activity in insulin digests. Thus the activity of three digests which started with 3·8, 5·0 and $4{\cdot}7 \times 10^{-4}$ T.U./ml. became 4·0, 3·3 and $5{\cdot}1 \times 10^{-4}$ T.U./ml. after 24, 24 and 10 hr. respectively.

The enzyme assay was done in a solution 5·55 M with respect to urea, and it is thus possible that enzyme-inhibitor complexes, if present, will be broken rapidly in this medium. Insulin was therefore digested in the presence of urea to see whether the breakdown was faster in such a medium.

Insulin (4·8 mg./ml.) was digested with 3×10^{-4} T.U. of chymotrypsin/ml. at pH 8·3 and 25° in three parts: (i) control without urea; (ii) in M-urea and (iii) in 5 M-urea. The N.P.N. was determined by precipitating 1 ml. with 5 ml. of 0·3 N-TCA and measuring the colour of the filtrate with the Folin-Ciocalteu phenol reagent. Fig. 1 shows the increase

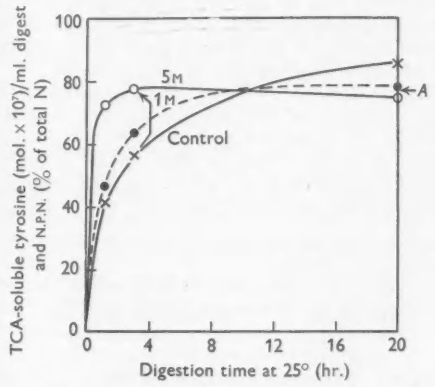

Fig. 1. Digestion of insulin by chymotrypsin. Control (×—×), in M-urea (●- - -●) and in 5 M-urea (○—○), as measured by colour with Folin reagent. *A* = point checked by nitrogen estimation.

during digestion of TCA-soluble material giving colour with this reagent. It has been found in separate experiments (see below) that the intensity of the blue colour developed in this way is directly proportional to the N content. An N determination in the 20 hr. control digest gave the value 77% N.P.N. (point *A*). Insulin is not, however, digested more rapidly in the presence of urea, the increased N.P.N. formation in Fig. 1 being due to the solubilizing effect of the urea lowering the efficiency of TCA. This was shown by adding urea to the TCA used to precipitate the control digest. By adding sufficient to simulate the conditions in the 5 M-urea experiment, the 'N.P.N.' of the control was raised as is shown in one instance by the arrow in Fig. 1. Addition of urea after filtration had no effect on the colour produced. In some tentative experiments to reduce the urea content of digest samples for injection, it was found that urea could be extracted readily from aqueous solution by *tert.*-amyl alcohol.

Addition of a solution of intact insulin to a chymotryptic digest which had reached 78% N.P.N. (1 ml. digest to 5 ml. 0·3 N-TCA) lowered the N.P.N. value to 39%, and was quickly followed by digestion which restored the N.P.N. value to 77%. Thus a high degree of enzyme activity remained. Moreover, the addition of peptides (derived from a chymotryptic digestion of insulin) to a solution of insulin did not affect its rate of digestion at pH 8 and 25° by chymotrypsin when compared with a control. Two experiments with added peptide : insulin ratios (by weight) of 0·28 and 0·72 were done.

If extra enzyme is added to a digest which has already become apparently stationary as regards N.P.N. formation, further formation of N.P.N. occurs at a slow rate. This is ascribed to the digestion of the core (mol.wt. 5000) which is normally precipitated by TCA (see Butler *et al.* 1950). It is clear from these experiments that the earlier opinion of Butler *et al.* (1950), that chymotrypsin is inhibited by an insulin digest, can no longer be sustained.

The effect of pH *on the rate of digestion of insulin by chymotrypsin*

The pH *optimum.* Insulin (2·15 mg./ml.) was digested with 3×10^{-4} T.U. of enzyme/ml. at 25° in buffers at various pH (measured by glass electrode), and samples taken at intervals for N.P.N. estimation (1 ml. digest to 5 ml. 0·3 N-TCA). The results are shown in Fig. 2. The optimum is near pH 8·6, but there is a second optimum near pH 9·5; series of digestions confirmed this observation. Resolution of the curve after 1·5 hr. digestion into two symmetrical curves gives the optima at about pH 8·2 and 9·7.

The effect of buffer. After 1·5 hr. digestion only a 1% difference in N.P.N. value was observed between a digest in pH 8·3 phosphate-citrate buffer and another in borate-hydrochloric acid buffer. Moreover, the buffers used over the whole pH range in Fig. 2 were borate-HCl or borate-NaOH, and in phosphate-citrate buffer it was shown that the digestion rate was lower at pH 9·0 than at pH 8·2 or 8·3.

Enzyme activity. Solutions of chymotrypsin at pH 7·6 and 9·9 in borate buffers as used above were found to have the same activity initially and after 6 hr. at 25°, and no absolute loss of activity was observed. Thus the double optimum is not related to any differential loss of activity at different pH by this enzyme.

Samples of digests prepared at pH 7·6 and 9·9 (on the opposite limbs of the pH-digestion curve, Fig. 2) were

coupled with 1-fluoro-2:4-dinitrobenzene according to the method of Sanger (1945), and a qualitative examination of the end groups by paper chromatography with *tert.*-amyl alcohol-water was made. The pattern, position and intensity of the yellow spots of dinitrophenylamino-acids were the same in both cases. Moreover, both digests had lost about 80% of their insulin activity after 5 hr. digestion at 25°.

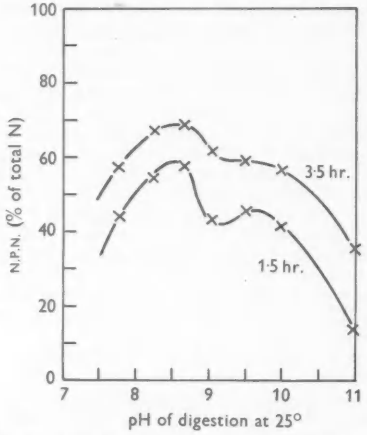

Fig. 2. Digestion of insulin by chymotrypsin at various pH values. Formation of N.P.N. after 1·5 and 3·5 hr. digestion at 25°.

As the activity of insulin is unaffected in 17 hr. at pH 9·9 and 25° as measured in a few standardized rabbits, it is unlikely that the second optimum is due to the presence of denatured or inactive insulin, especially as the two optima are more apparent after 1·5 hr. digestion than after 3 hr.

Enzyme specificity in the digestion of insulin by chymotrypsin

End groups. Chymotrypsin favours the fission of peptide, amide or ester bonds liberating the carboxyl group of tryptophan, tyrosine, phenylalanine or methionine (Bergmann & Fruton, 1937; Fruton & Bergmann, 1942; Kaufman, Neurath & Schwert, 1949; Neurath & Schwert, 1950). Such rules have not yet been proven for proteins, though a start has been made with pepsin by Desnuelle & Casal (1948), using Sanger's (1945) fluorodinitrobenzene method. This, however, can only identify amino-acids which provide the —NH— group of the peptide bond which is split. This method, together with that of Mills (1950) and paper chromatography of the dinitrophenyl (DNP) amino-acids with *tert.*-amyl alcohol-water, have been used qualitatively on chymotryptic digests of insulin.

In the TCA-precipitable cores (see Butler *et al.* 1950) the following end groups were found: (1) DNP-glycine, 2–3 residues; (2) DNP-valine, 1 residue. DNP-leucine has also been found. In the TCA-soluble peptides the DNP derivatives of leucine, valine, phenylalanine, alanine, glycine, glutamic acid, threonine and tyrosine were found, the proportions decreasing in that order, the tyrosine being present only as a trace. Now insulin has two glycine and two phenylalanine terminal groups per mol.wt. 12,000 (Sanger, 1945), so that these would be expected in the end groups of the products. These findings support the view of Bergmann & Fruton that chymotrypsin splits bonds on the carboxyl side of aromatic amino-acids.

The 'all-or-none' character of the degradation of insulin by chymotrypsin

From electrophoretic studies of peptic digests of ovalbumin, Tiselius & Eriksson-Quensel (1939) postulated that no products intermediate between ovalbumin and the final products appeared during the digestion, i.e. the reaction for a given molecule was completed apparently in one operation.

In the digestion of insulin, this has been studied by measuring (*a*) the average number of bonds broken per degraded molecule; (*b*) the tyrosine released in the form of TCA-soluble peptides; (*c*) the proportions of TCA-precipitable core and TCA-soluble peptides formed; and (*d*) the activity

Table 1. *Number of bonds broken during the chymotryptic digestion of insulin*

Percentage of intact insulin remaining (*A*)	Percentage of molecules degraded (100 − *A*)	Average number of bonds broken in all molecules (Van Slyke) (*B*)	Average number of bonds broken per degraded molecule (100*B*/100 − *A*)
100	0	0	—
92–93*	7–8	0·8	10–11·4
55–60*	38–44	5·3	12–13·9
30–35*	65–70	7·0	10–11·5
34	66	8·3	12·4
26	74	10·4	14·0
11·5	88·5	12·3	13·9
4·4	95·6	14·2	14·8

* These values were obtained by measurement of the N.P.N. of the digest, and the knowledge that the amount of core formed is 75–100% of the amount of N.P.N. The other values were obtained by quantitative assay of activity (by Boots Pure Drug Co. Ltd.) of the TCA precipitates at stages during digestion, and agree well with the amount of precipitate formed on bringing the digest to pH 5·4, the isoelectric point of insulin.

remaining in the digest, and comparing it with that expected in a random fission of the enzyme-specific bonds, assuming that any single fission inactivates the hormone. This assumption has not been proven but, at least, none of the products of this digestion has any hypoglycaemic activity (Phillips, 1951).

Number of bonds broken. Amino nitrogen determinations were made at intervals during the digestion by Dr J. M. L. Stephen. Table 1 shows the relation of this measurement to the number of molecules degraded. Every molecule attacked has therefore had almost the full number or bonds broken in it irrespective of the time of sampling.

Table 2. *Experimental and calculated loss of insulin activity in samples of insulin digested by chymotrypsin*

Percentage insulin activity remaining	Average number of bonds split per molecule (n)	Calculated percentage of molecules still intact
100	0	100
92	1	37
84*	2	13·5
77*	3	5
34	8	0

* These are interpolated from the experimental values from the known shape of the isoelectric precipitate-time of digestion curves, and the knowledge from the assays that this precipitate accounts for all activity remaining in the digests. Interpolation was necessary to keep n small compared with 15, the maximum fission.

If the assumption is made that the fission of a single peptide bond in insulin by chymotrypsin causes inactivation of that molecule, then a random degradation process, which splits say one bond in each of fifteen molecules, will achieve far more inactivation than a process splitting fifteen bonds in one molecule before proceeding to deal with another molecule. How does the fall in activity of the digest compare with the calculated rate for the random process? It is assumed too that there are fifteen specific bonds per molecule (this being the total number of aromatic amino-acid residues and approximately the number of bonds split in chymotryptic digests of insulin reaching 60–70% N.P.N.). Then the number of intact molecules remaining, when the number of bonds broken per mol. is still small compared with fifteen, is given by the Poisson distribution

$$P = \frac{n^m}{m!}\epsilon^{-n},$$

where m = actual number of bonds split in a given molecule (when $m = 0$, the molecule is intact and active), and n = the average number of bonds split in all the molecules.

Table 2 shows this value as a percentage for a series of degrees of digestion from $n = 0$ to $n = 8$. The loss of activity in the random process is far more rapid than occurs in practice (column 1), so that with the assumption made, the process is far from random and a much smaller proportion of the molecules is attacked. Once attacked, each molecule is totally disrupted.

Tyrosine released. The tyrosine in the TCA-soluble peptides at various stages during the digestion was determined in 6 N-hydrochloric acid hydrolysates and in unhydrolysed samples. Table 3 shows the results of the former analyses and Fig. 3

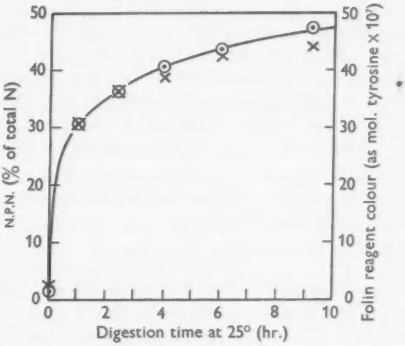

Fig. 3. Correlation of N.P.N. formed (⊙) and the colour given by the N.P.N. samples with the Folin-Ciocalteu reagent (×) during the chymotryptic digestion of insulin.

the parallelism between the latter analyses and the measured N.P.N. at the same stages of digestion. These results show that the tyrosine released per molecule attacked is, like the amino groups, a constant throughout the digestion.

Core and peptide proportions. The relative proportions of the two classes of products of the digest —the core and the TCA-soluble peptides—remain nearly constant throughout the digestion, as shown in Fig. 4. The undigested insulin was estimated by adjusting the pH of the digest to the isoelectric point of insulin and centrifuging off the precipitate.

Table 3. *Tyrosine content of the TCA-soluble peptides after acid hydrolysis*

N.P.N. as percentage of total N	Total peptide N taken (mg.)	Tyrosine N in peptides (μg.)	$\frac{\text{Tyrosine N} \times 100}{\text{Total peptide N}}$
30·4	1·57	118·4	7·55
36·3	0·615	45·0	7·3
40·8	0·56	45·2	8·0
43·7	0·50	32·0	6·4
47·5	0·43	31·2	7·2

The core in the clear centrifugate was then precipitated with TCA. The relation alters towards high values owing to the appreciable secondary digestion of the core. Alternatively, a plot of the percentage of core or of peptides formed against the percentage of insulin remaining in the digest (as measured by the amount of isoelectric precipitate) also gives a straight line.

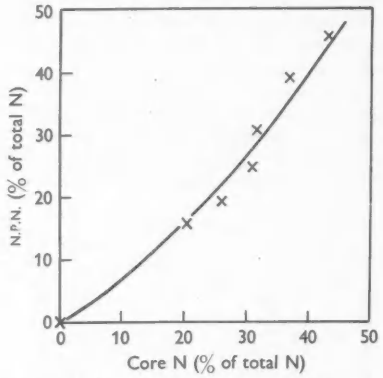

Fig. 4. The relation between the amount of core nitrogen and TCA-soluble peptide nitrogen, during the chymotryptic digestion of insulin.

DISCUSSION

The results show that very little enzyme activity is lost during the chymotryptic digestion of insulin, and that the rapid slowing down of this digestion when the N.P.N. is 40–50% (see Butler *et al.* 1948) is not due to the spontaneous inactivation of the enzyme, nor to its inhibition by the products. In this connexion many cases of inhibition of an enzyme by the products of its action have been reported, e.g. urease by ammonia (Laidler & Hoare, 1949).

The double optimum of digestion described above is most simply interpreted as due to chymotrypsin containing more than one enzyme or due to insulin being more than one protein. Kunitz & Northrop (1935) digested casein with α-chymotrypsin, and if a smooth curve is drawn through their formol titration points a double optimum results, which is not entirely eliminated by adding the points from nitrogen determinations. It is known, however, that casein consists of two major and one minor component (Gordon, Semmett, Cable & Morris, 1949) and the net charge per molecule on the major components will be considerably different at pH between 7 and 10. On the other hand, Rocha e Silva & Andrade (1943) found a very marked double optimum in the digestion of a single substrate, *N*-benzoyl-L-arginineamide by activated papain, but in this case it is known that papain consists of more than one enzyme.

The chymotrypsin used here was found (by Dr J. M. Creeth) on electrophoresis at pH 8 to have an isoelectric point lower than pH 8 and to have about 25% of a minor component, migrating faster and also having an isoelectric point on the acid side of pH 8. When titrated in the presence of sodium dodecyl sulphate a precipitate formed at a pH lower than 4·9, suggesting that the isoelectric point is the same as that determined by Kunitz & Northrop (1935) from the migration of coated collodion particles. Neurath & Schwert (1950), however, accept the values of 9·5 for the isoelectric point of α-chymotrypsinogen and 8·1–8·6 for α-chymotrypsin measured by Anderson & Alberty (1948) and Kubacki, Brown & Laskowski (1949). They also report that α-chymotrypsinogen precipitates at pH 5·4 in the presence of dodecyl sulphate and suggest the isoelectric point may be influenced by a dimerization. However, it is curious to note that the chymotrypsin *B* and its zymogen prepared by Brown, Shupe & Laskowski (1948) had isoelectric points at 4·7 and 5·2, respectively, and had a specificity indistinguishable from that of chymotrypsin (Fruton, 1948). It seems that if the insulin double optimum is to be explained by the presence of two components in the chymotrypsin, then the minor enzyme components would have to be very much more active than the major component in order to achieve the similar rates of degradation actually observed.

Some samples of crystalline insulin contain a hyperglycaemic factor (Sutherland & Cori, 1948), but the material used here has not been tested for this. However, the usual quantity of the factor is estimated at not more than 5% of the whole, and as the optima indicate the liberation of more than 50% of the nitrogen as N.P.N. at both optima, the presence of such a small amount could not account for the result. On the other hand, some evidence of the inhomogeneity of insulin has been obtained (Phillips, unpublished) by chromatography of the 2:4-dinitrophenyl derivative of insulin, containing the theoretical number of 19 added groups per mol. Three components soluble in alkaline methyl ethyl ketone were obtained, accounting for 40% of the insulin. These components had different proportions of dinitrophenyl groups in them. While this is yet inconclusive it seems the most likely explanation of the double optimum observed in the digestions.

The specificity of chymotrypsin towards synthetic peptide substrates is now quite well defined (Neurath & Schwert, 1950), and as there is no tryptophan or methionine in insulin, the only specific residues involved are those of tyrosine and phenylalanine (assuming these rules apply to proteins). The specificity revealed in the chymotryptic digestion of insulin indirectly supports the results with synthetic peptides and suggests that leucine

and valine, with long aliphatic side chains, are favoured components on the 'unspecific' side of the peptide bond split. However, the complete description of the specificity towards a protein must await either an elucidation of the amino-acid sequence in the neighbourhood of these aromatic residues or the introduction of a reagent for the identification of the other partner of the specific peptide bond.

The results show that every molecule attacked by the enzyme undergoes thirteen or fourteen fissions even though there may be a vast excess of intact insulin molecules present. All this degradation may be achieved within the lifetime of the first enzyme-insulin complex formed, or the affinity between intermediates (i.e. molecules of size intermediate between insulin and the core) and the enzyme is so much greater than the insulin-enzyme affinity so that the intermediates are preferentially attacked. This is likely on the grounds that denatured proteins are more readily degraded than the native forms. The measurements made here cannot definitely decide whether the fourteen bonds are split in one catastrophic process or as a result of successive approaches of enzyme to partially degraded material.

Although any fission of insulin (except the removal of amide groups) causes inactivation and suggests that the whole of the insulin molecule is essential for activity, it is more likely, in view of the essential nature of only some of the tyrosine groups, a few of the carboxyl groups and the different rates of inactivation per bond split by different enzymes, that there is a specially active group in the molecule. Thus far no degrading agent has come to light which avoids this grouping. From this one infers that many bonds might be broken without affecting the activity, and in such a degradation even a random fission of bonds would cause a much slower rate of inactivation than in the case calculated in Table 2, where the fission of any single bond is assumed to cause inactivation. However, in any random case, the presence of intermediates should be demonstrable. No exhaustive search has been made for such intermediates, and it has been assumed that protein precipitating at the isoelectric point of insulin and having a weight activity approximately equal to that of insulin is actually intact insulin. Moreover, it has been shown that the core is homogeneous (Butler *et al.* 1950). A complication follows here in that insulin may have a molecular weight of only 6000 (Fredericq & Neurath, 1950). If this is so, one has to assume either that the core is an association of smaller units to give a mol.wt. of 5000, or that insulin contains two types of molecule, one being only slightly degraded to give the core and the other completely degraded to trichloroacetic acid-soluble peptides, averaging pentapeptides. The core is probably derived from that end of the peptide chains of insulin bearing the free α-amino groups, and the fact that it is inactive (Phillips, 1951) and that insulin which has lost only one or two alanine residues from the carboxyl ends of the peptide chains (Lens, 1949) is also inactive, strongly suggests that the end bearing the free carboxyl groups of insulin must be left intact if activity is to be retained.

However, if it is assumed that the fourteen fissions occur within the lifetime of the enzyme-insulin complex, some sort of lability even in this combination seems to be required, since there is only one site on each enzyme molecule responsible for causing fission. At least, this is the simplest interpretation of the finding by Jansen, Nutting, Jang & Balls (1949) that one molecule of di*iso*propyl fluorophosphonate (DFP) inactivates one molecule of chymotrypsin of mol.wt. 27,000. This may merely mean that an essential anchorage is denied the enzyme while its active centres remain unaffected. However, the fact that the fission of a substrate as small as L-tyrosine ethyl ester is also prevented by treatment of the enzyme with DFP implies that such anchorage is very near if not coincident with the active point of the enzyme.

SUMMARY

1. Throughout the digestion of insulin by chymotrypsin, the enzyme remains almost fully active, and the digestion rate is unaffected by the presence of urea or the products of the digestion.

2. The pH-digestion rate curve for this proteolysis has two optima, at pH 8·6 and 9·5.

3. The double optimum is not due to inactivation of part of the insulin or the enzyme, and the bonds split at pH 7·6 and 9·9 appear to be the same.

4. Insulin is inactivated at the same rate at pH 7·6 and 9·9 by chymotrypsin.

5. The amino groups revealed by the proteolysis belong mostly to amino-acids other than tyrosine and phenylalanine, and together with the distribution of tyrosine and phenylalanine among the products give indirect support to the specificity rule suggested for this enzyme by Bergmann & Fruton (1937).

6. The degradation has the character of an 'all-or-none' reaction as postulated by Tiselius & Eriksson-Quensel (1939). Every molecule degraded has undergone the fission of thirteen or fourteen bonds irrespective of the number of intact insulin molecules present.

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On the Cyanide Inactivation of Succinic Dehydrogenase and the Relation of Succinic Dehydrogenase to Cytochrome *b*

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Unlike most oxidizing enzymes, the dehydrogenases were considered to be insensitive to cyanide, until Dixon & Keilin (1936) discovered that xanthine dehydrogenase is slowly but irreversibly inactivated by cyanide. In fact, the cyanide inactivation of xanthine dehydrogenase is in a class of its own. The usual type of cyanide inhibition of oxidizing enzymes is instantaneous, readily reversible and only partial, a definite percentage inhibition to each cyanide concentration. In the case of xanthine dehydrogenase, the inhibition is irreversible and it proceeds to completion if, prior to the addition of substrate, the enzyme is allowed to remain with cyanide for a sufficiently long time.

The treatment which Dixon & Keilin (1936) used with xanthine dehydrogenase was applied to a number of enzymes, including succinic dehydrogenase, by Leloir & Dixon (1937) and no inactivation by cyanide was obtained. This was later confirmed by Potter (1941), who found that the enzyme system responsible for the reduction of cytochrome *c* by succinate was not affected by incubation with cyanide. On the other hand, using a higher cyanide concentration, Banga & Porges (1938) and recently Slater (1949*b*) reported some inhibition of succinic dehydrogenase by cyanide.

In the present investigation it is shown that succinic dehydrogenase is slowly and irreversibly inactivated by cyanide. It is also shown that the enzyme can be effectively protected from the action of cyanide by succinate and sodium dithionite; dicarboxylic acids other than succinic are, however, incapable of giving any protection. A preliminary account of some of these findings has appeared elsewhere (Tsou, 1951*a*).

MATERIALS AND METHODS

Enzyme preparations. The heart-muscle and kidney preparations (Keilin & Hartree, 1947; Slater, 1949*b*) containing the succinic dehydrogenase-cytochrome system were prepared as previously described (Tsou, 1951*b*).

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Reagents. Solutions of cyanide, cysteine and pyrophosphate were prepared and neutralized immediately before use. Cystine solution was prepared according to Hopkins & Morgan (1938).

Succinic oxidase and cytochrome oxidase activity was estimated essentially as described by Slater (1949*a*, *b*).

Succinic dehydrogenase activity was estimated by the Thunberg technique at 38°. Unless otherwise specified, final concentrations were: phosphate buffer, pH 7·3, 0·1 M; succinate, 0·04 M; enzyme, 1–2 mg. fat-free dry weight; methylene blue (MB), 0·125 mM, or dichlorophenol indophenol, 0·5 mM. 90% reduction of the dye was taken as the end point, and activity was expressed in terms of Q_{MB} as defined by Corran, Dewan, Gordon & Green (1939) on the hydrogen basis.

Inactivation by cyanide. The correct amount of neutralized 0·1 M-cyanide to give the required concentration was added to heart-muscle or kidney preparations, previously brought to the desired temperature, in 0·1 M-phosphate buffer, pH 7·3. After thorough mixing, the sample was incubated in a bath of constant temperature. Portions were withdrawn at suitable intervals and the activity was estimated as described above. Controls were carried out where the enzyme was incubated with the same concentration of KCl instead of KCN.

Freeing the inactivated enzyme from cyanide. The enzyme preparation which had been incubated with cyanide for a specified period was dialysed first against running tap water for 4 hr. followed by two changes of 0·1 M-phosphate buffer of pH 7·3. It was allowed to remain in the second change of buffer overnight. As a control, a sample of the same enzyme preparation was incubated with KCl instead of KCN and then dialysed simultaneously. When a protective agent was also present during the incubation, the cyanide-treated enzyme preparation was first dialysed against a solution of the protective agent and then dialysed against running tap water and buffer as described above.

RESULTS

Course of inactivation. The inactivation of heart-muscle succinic dehydrogenase by cyanide is shown in Fig. 1. The succinic dehydrogenase in the kidney preparation is also affected by cyanide, although at a slower rate than the heart-muscle enzyme. Fig. 2 compares the cyanide inactivation of succinic dehydrogenase in these two preparations. Unlike the Ogston & Green (1935) preparation used by Leloir & Dixon (1937), both the heart-muscle and kidney preparations are perfectly stable at the temperature used (38°) in the absence of cyanide.

Table 1 shows that, as in the case of xanthine dehydrogenase, the effect of cyanide on succinic dehydrogenase was only produced after it had acted upon the enzyme for a definite time in the absence of substrate (tube 3). No inactivation was obtained when cyanide was introduced together with substrate and acceptor (tube 4), nor when the enzyme was incubated with cyanide in presence of the substrate (tube 2).

In addition to cyanide, the action of other respiratory inhibitors such as carbon monoxide,

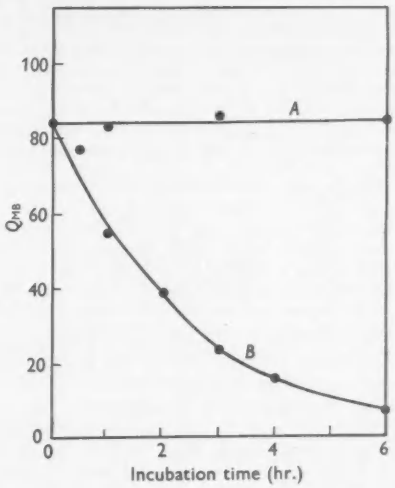

Fig. 1. Inactivation of succinic dehydrogenase by cyanide. Heart-muscle preparation in 0·1 M-phosphate buffer, pH 7·3, was incubated at 38°, alone (curve *A*) and with 0·005 M-cyanide (curve *B*). Portions were taken at suitable intervals for activity estimation.

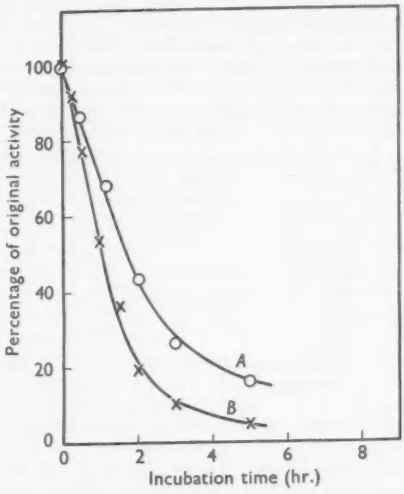

Fig. 2. Inactivation of heart-muscle and kidney succinic dehydrogenase by cyanide. Heart-muscle preparation (curve *B*) and kidney preparation (curve *A*), both in 0·1 M-phosphate buffer, pH 7·3, were incubated with 0·01 M-cyanide at 38°. Portions were taken at suitable intervals for activity estimation. In order to compare the two curves, activity of both preparations is expressed in percentage of original activity. Both preparations were perfectly stable in absence of cyanide.

Table 1. *Inactivation of succinic dehydrogenase by cyanide*

(Experiments were carried out in Thunberg tubes, one of them (no. 4) with two hollow stoppers. The tubes contained 0·1 ml. dehydrogenase (enz.); 0·04 M-succinate (suc.); 0·3 mM-methylene blue (MB); 0·02 M-KCN, neutralized; and 0·1 M-phosphate buffer, pH 7·3 (buf.). When KCN in solid form was added (5 mg.) in one of the hollow stoppers, an equivalent amount of H_2SO_4 (0·7 ml., 0·1 N) was added to the other. Volumes were made up to 4 ml. with water. After being allowed to stand at room temperature for 4 hr. the tubes were evacuated and warmed to 38°. The contents of the main tubes and the hollow stoppers were mixed and the reduction time measured.)

Tube no.	Main tube	Hollow stoppers A	Hollow stoppers B	Reduction time min.	Reduction time sec.
1	Enz. + buf.	MB + suc.	—	2	25
2	Enz. + buf. + HCN + suc.	MB	—	2	30
3	Enz. + buf. + HCN	MB + suc.	—	28	—
4	Enz. + buf.	MB + suc. + H_2SO_4	KCN	2	20

sulphide and azide on the enzyme was also studied. None of them, however, shows any action on the enzyme even after prolonged incubation either in presence or in absence of the substrate.

Irreversibility. When the cyanide-treated muscle preparation was freed from cyanide by dialysis the cytochrome-oxidase activity was completely restored, whereas the succinic dehydrogenase remained inactive. This is shown in Table 2. In

Table 2. *The activity of cyanide-treated heart-muscle preparation*

(Heart-muscle preparation was incubated with 0·01 M-cyanide at pH 7·3 for 18 hr. at room temperature. Cyanide was removed by dialysis at the end of incubation. As a control the same preparation was incubated with 0·01 M-KCl and dialysed simultaneously. Activity of succinic dehydrogenase (Suc.D.) is expressed as Q_{MB}; activities of succinic oxidase (Suc.O.) and cytochrome oxidase (Cyt.O.) are expressed as Q_{O_2} in μl./mg. fat-free dry wt./hr.)

System	Substrate	Added cytochrome c (mM)	Activity Control	Activity Cyanide treated
Suc.D.	Succinate	—	79	1
Suc.O.	Succinate	—	402	Nil
Suc.O.	Succinate	0·06	575	Nil
Cyt.O.	Ascorbate	—	25	27
Cyt.O.	Ascorbate	0·06	1850	1720
Cyt.O.	*p*-Phenylenediamine	—	460	480
Cyt.O.	*p*-Phenylenediamine	0·06	1490	1400

order to ensure the complete removal of cyanide, the cyanide-treated enzyme preparation was dialysed as described before and then incubated with a large excess of methaemoglobin at room temperature for 24 hr. Methaemoglobin is known to have a high affinity for cyanide and should have reacted with any remaining cyanide. Estimation by the Thunberg method revealed that 24 hr. incubation with excess methaemoglobin had no effect whatsoever on the inactivated succinic dehydrogenase of the cyanide-treated and dialysed preparation as is shown in Table 3.

Table 3. *Effect of methaemoglobin on the cyanide-inactivated succinic dehydrogenase*

(Conditions of cyanide treatment and dialysis as in Table 2. Control with KCl as usual.)

Treatment	Succinic dehydrogenase (Q_{MB}) Control	Succinic dehydrogenase (Q_{MB}) Cyanide treated
Before treatment	79	79
After incubation with KCl and KCN respectively	79	1·5
After dialysis	77	1·2
After incubation with methaemoglobin at room temperature for 24 hr.	78	1·4

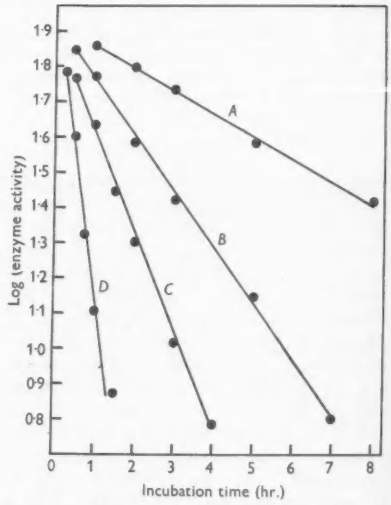

Fig. 3. Relation between the concentration of cyanide and the rate of inactivation of succinic dehydrogenase. Heart-muscle preparation was incubated at 22° with 0·005 M (*A*), 0·01 M (*B*), 0·02 M (*C*), 0·05 M (*D*) cyanide. Enzyme activity was expressed in Q_{MB}.

Inactivation kinetics. It was found that the reaction between succinic dehydrogenase and

cyanide followed the first-order equation 1 when cyanide was in excess over the enzyme

$$-\frac{d \ln E}{dt} = k[\mathrm{KCN}], \qquad (1)$$

where E is the concentration of the enzyme. A plot of log E against time gave a series of straight lines at different cyanide concentrations (Fig. 3). It follows from (1) that the time for 50 % inactivation ($t_{0.5}$) is given by

$$t_{0.5} = \frac{\ln 2}{k\,[\mathrm{KCN}]}. \qquad (2)$$

Hence a plot of $t_{0.5}$ against the reciprocal of cyanide concentration gives a straight line with a slope of $\ln 2/k$ (Fig. 4). The values of the reaction velocity constant k at 22° and 38°, calculated from the slopes of the straight lines obtained in Fig. 4 were,

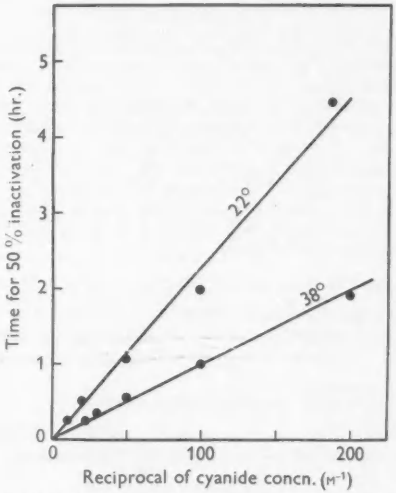

Fig. 4. Relation between the concentration of cyanide and the rate of inactivation of succinic dehydrogenase. Time for 50 % inactivation at 22° and 38° is plotted against reciprocal of cyanide concentration.

respectively, 0·51 and 1·15 l./mol./min. The average value obtained from Fig. 3 for 22° was 0·52. The energy of activation calculated from the Arrhenius equation was 9500 cal.

Effect of pH *of incubation.* Table 4 shows that change of pH of incubation from 6·5 to 8 had no effect on the rate of inactivation. As a difference of one pH unit in this region reflects approximately a tenfold difference in free cyanide ion concentration, the fact that a change in pH from 6·5 to 8 did not affect the rate of inactivation of the enzyme could best be explained by assuming the reaction as one between hydrocyanic acid and the enzyme.

Table 4. *Inactivation of succinic dehydrogenase at different* pH *of incubation*

(A heart-muscle preparation was prepared in the usual way. The final pH 5·5 precipitate was suspended in an equal volume of water, divided into three portions and each was then mixed with one-quarter its volume of 0·25 M-phosphate buffer of pH 6·5, 7·3 and 8·2, respectively. Potentiometric measurements with a glass electrode gave pH 6·43, 7·17 and 8·04 respectively for the three final preparations. The incubation was carried out in 2 ml. syringes. Each syringe received 1·8 ml. of the enzyme mixed with 0·2 ml. of 0·1 M-cyanide. The needles of the syringes were inserted into rubber bungs to prevent the escape of hydrocyanic acid vapour. KCl controls were carried out as usual. Activity estimations were carried out at pH 7·3. The Q_{MB} values after 2 and 4 hr. incubation at room temperature are listed as follows.)

	pH of incubation					
	6·43		7·17		8·04	
Incubation time (hr.)	KCl	KCN	KCl	KCN	KCl	KCN
0	78	79	82	81	80	80
2	80	41	78	40	80	38
4	79	17	81	18	79	14

Protection of the enzyme from inactivation. The protection of succinic dehydrogenase by succinate has already been shown in Table 1. Table 5 shows that 0·01 M-succinate completely protects the enzyme from the action of cyanide. The degree of protection by succinate depends upon its concentration as is shown in Fig. 5. Although a concentration of 2 mM-succinate was enough to give complete protection, a measurable degree of protection was given by as little as 0·02 mM.

In marked contrast to xanthine dehydrogenase, which can be protected from cyanide inactivation by several chemically related purine compounds, no other dicarboxylic acids, except succinic acid, were found to be effective in the case of the succinic enzyme. Malonate and fumarate, known to have a high affinity for the enzyme, were both ineffective; so were pyrophosphate, cystine and cysteine. This is shown in Tables 6–8.

Apart from succinate, only sodium dithionite was able to protect succinic dehydrogenase from the action of cyanide, although dithionite oxidized by aeration was ineffective. This is shown in Table 9.

Relation of succinic dehydrogenase to cytochrome b

A freshly prepared heart-muscle preparation in 0·1 M-phosphate buffer, pH 7·3, was incubated with 0·01 M-cyanide for 24 hr. at room temperature. At the end of the incubation, this preparation had a Q_{MB} of 1·4 which was less than 2 % of the original activity ($Q_{MB} = 86$). On the addition of a few drops of dihydrocozymase, there appeared a strong

reduced cytochrome *c* band together with a somewhat weaker reduced *b* band. Moreover, the cytochrome *b* present in the cyanide-treated enzyme preparation can also be reduced by the addition of a few drops of an untreated enzyme followed by succinate, although this reduction is rather slow.

Table 5. *Protection of succinic dehydrogenase from the action of cyanide by succinate*

(To 8 ml. of heart-muscle preparation were added 1 ml. of 0·1 M-succinate and 1 ml. of 0·1 M-cyanide. A second 8 ml. portion of the enzyme received 1 ml. of water and 1 ml. of 0·1 M-cyanide; and a third 8 ml. portion, 1 ml. water and 1 ml. of 0·1 M-KCl. The three mixtures were allowed to stand at room temperature for 18 hr., and then dialysed as previously described.)

Treatment		Activity (Q_{MB})
Before treatment		75
After incubation with:	KCN + succinate	75
	KCN alone	1
	KCl alone	74

If succinic dehydrogenase were identical with cytochrome *b*, it would be possible, on incubation with cyanide, to observe a parallel relation between the decrease in the succinic dehydrogenase activity and the intensity of the α-band of reduced cytochrome *b*. However, careful matching of the intensity of the α-band of reduced *b* in heart-muscle preparation treated with cyanide for different times failed to reveal any parallel relation. The matching was carried out against a standard of normal heart-muscle preparation with a low dispersion microspectroscope provided with a double-wedged trough. Even the inactivated preparation, with an activity of less than 2 % that of the original, could still be reduced by succinate, although it took some 45 min. for the reduced *b* band to appear, with an intensity about 15 % that of the original. This was not surprising as with this slow rate of reduction the autoxidation of cytochrome *b* in the presence of cyanide must also be taken in consideration. In the complete absence of air, the reduction of cytochrome *b* by succinate

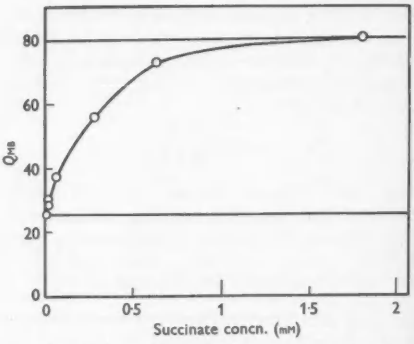

Fig. 5. Protective effect of different concentrations of succinate on the rate of cyanide inactivation of succinic dehydrogenase. The activity is plotted against succinate concentration. The upper horizontal line represents the Q_{MB} obtained without cyanide, thus corresponding to complete protection, while the lower line shows the Q_{MB} obtained after 3 hr. incubation with 0·01 M-cyanide without succinate and thus corresponds to zero protection. The intermediate points are the Q_{MB} obtained after similar incubation with cyanide in presence of different succinate concentrations.

Table 6. *Effect of malonate on the inactivation of succinic dehydrogenase by cyanide*

(Portions of enzyme preparation were incubated with 0·01 M-cyanide (1*b*, 2*b* and 3*b*) or with 0·01 M-KCl (1*a*, 2*a* and 3*a*) at room temperature. Portions 2*a* and 2*b* also contained 0·017 M-malonate and portions 3*a* and 3*b*, 0·05 M-malonate. At suitable intervals, 0·2 ml. samples were taken from each portion for activity estimation.)

Time of incubation (hr.)	Activity (Q_{MB})						Percentage inactivation in presence of malonate (M)		
	1*a* KCl	1*b* KCN	2*a* KCl	2*b* KCN	3*a* KCl	3*b* KCN	None	0·017	0·05
0	77	76	46	47	29	27	—	—	—
1	76	54	46	34	26	20	29	28	29
2	76	36	50	23	26	13	53	52	54
4	78	20	46	12	28	8	74	75	72

Table 7. *Effect of fumarate on the inactivation of succinic dehydrogenase by cyanide*

(Conditions as in Table 6, except that instead of malonate, portions 2*a* and 2*b* contained 0·017 M-fumarate and portions 3*a* and 3*b*, 0·05 M-fumarate.)

Time of incubation (hr.)	Activity (Q_{MB})						Percentage inactivation in presence of fumarate (M)		
	1*a* KCl	1*b* KCN	2*a* KCl	2*b* KCN	3*a* KCl	3*b* KCN	None	0·017	0·05
0	76	77	74	75	76	74	—	—	—
1	79	54	76	50	76	52	31	34	30
2	78	37	77	36	72	35	52	52	53
4	78	20	75	20	73	21	74	73	72

Table 8. *Effect of pyrophosphate, cysteine and cystine on the inactivation of succinic dehydrogenase by cyanide*

(The enzyme was incubated with 0·01 M-cyanide plus 0·01 M-pyrophosphate, 0·004 M-cysteine or 0·002 M-cystine for 6 hr. at room temperature. 0·2 ml. portions were taken for activity estimation at the end of incubation. Controls with KCl were carried out.)

	Activity (Q_{MB})	
	KCl	KCN
No addition	81	4·5
Pyrophosphate	74	4·0
Cysteine	72	3·2
Cystine	60	3·0

Table 9. *Effect of sodium dithionite on the inactivation of succinic dehydrogenase by cyanide*

(A heart-muscle preparation (4 ml.) was placed in the main tube of each of three Thunberg tubes each provided with two hollow stoppers. Solid KCN (0·5 mg.) was placed in one limb of each stopper and an equivalent amount of H_2SO_4 in the other. To the first tube was added 1 ml. of water, to the second, 1 ml. of a freshly prepared solution containing 10 mg. dithionite and to the third, 1 ml. of the same dithionite solution thoroughly aerated until it no longer had any reducing power. The three tubes were then evacuated, washed once with O_2-free nitrogen and again evacuated. The contents of the main tubes and hollow stoppers of the three Thunberg tubes were then mixed and allowed to stand for 18 hr. at room temperature. At the end of incubation, spectroscopic observations showed the presence in tube no. 2 of the cytochrome a_3-cyanide complex which indicated that dithionite had not combined with cyanide, the former being in excess. The three tubes were opened and dialysed until free from both cyanide and sodium dithionite.

Tube no.	Incubated with	Activity (Q_{MB}) after dialysis
Control	KCl	81
1	KCN alone	1
2	KCN + $Na_2S_2O_4$	82
3	KCN + aerated $Na_2S_2O_4$	2

in the cyanide-treated heart-muscle preparation, although extremely slow, eventually reached completion. Cytochrome *b* thus reduced was readily oxidized by methylene blue.

The pyrophosphate inhibition of succinic dehydrogenase

Leloir & Dixon (1937) expressed the view that pyrophosphate acts as an inhibitor of succinic dehydrogenase in the same way as malonate, i.e. competing with succinate for the enzyme by virtue of its two adjacent acid groups. In the present study, inhibition of succinic dehydrogenase by pyrophosphate in the presence of different pyrophosphate and succinate concentrations was measured by the Thunberg method. The results of measurements are plotted by Lineweaver and Burk's method in Fig. 6. They show conclusively that the pyrophosphate inhibition of succinic dehydrogenase is competitive in nature.

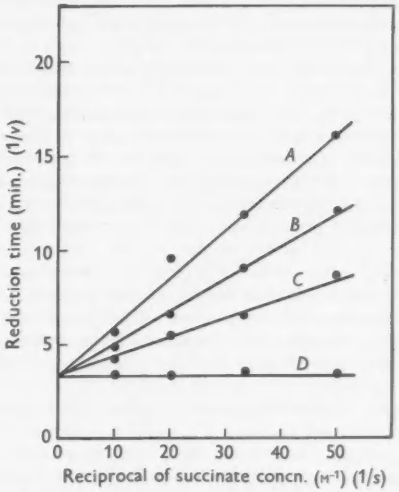

Fig. 6. The pyrophosphate inhibition of succinic dehydrogenase. The reciprocal of activity, in reduction time, is plotted against the reciprocal of substrate concentration. The fact that the four lines, representing different pyrophosphate concentrations (curves *A*, *B*, *C* and *D* represent 0·02, 0·0125, 0·0075 M and no pyrophosphate respectively), meet the ordinate at the same point shows that the inhibition is competitive.

DISCUSSION

Strictly speaking, the term 'succinic dehydrogenase' applies to the enzyme which is specifically concerned in the activation of succinate and probably acts also as the immediate hydrogen or electron acceptor. However, in fact, this term is generally used to denote that part of the succinic oxidizing enzyme system which is responsible for the catalysis of the anaerobic oxidation of succinate by dyes, such as methylene blue or dichlorophenol indophenol. Some workers even use the term succinic dehydrogenase when they are actually referring to the system that catalyses the aerobic oxidation of succinate. It seems most desirable, therefore, to emphasize here that in the present work the term 'succinic dehydrogenase' is used to denote the enzyme which is responsible for the specific activation of succinate and which, as evidence to be summarized later will show, also acts as the immediate hydrogen or electron acceptor. The term 'succinic dehydrogenase system', on the other hand, is used whenever confusion with the former term is likely to arise to denote the system which catalyses the anaerobic oxidation of

succinate by dyes. This system may include, besides succinic dehydrogenase, cytochrome *b* (Slater, 1949*b*). The differentiation between these two terms is necessary in view of the evidence presented in this paper which shows that succinic dehydrogenase is not identical with cytochrome *b*.

The protection of an enzyme from destruction by its substrate is a well known phenomenon. However, in most cases, protection is also given by compounds which are closely related in chemical structure to the substrate and are competitive inhibitors. In these cases the simple explanation that both the substrate and the closely related substances can combine with the enzyme thus blocking the active centre of the enzyme from attack by the destructive agents is adequate. In the present case the failure of dicarboxylic acids other than succinic acid and of pyrophosphate to protect succinic dehydrogenase from destruction by cyanide indicates that an altogether different explanation is necessary.

An alternative explanation is that cyanide reacts only with the oxidized form of the enzyme. Once the enzyme is reduced it no longer reacts with cyanide. This explanation also makes it necessary to place succinic dehydrogenase actually in the electron-transferring chain of the oxidation of succinate, that is to say, the enzyme, apart from being responsible for the activation of succinate, is also actually its immediate electron or hydrogen acceptor. Evidence in support of this view is as follows:

(1) Although 2×10^{-3} M-succinate is sufficient to protect the enzyme from the action of cyanide, and a measurable degree of protection can be obtained with a succinate concentration as low as 2×10^{-5} M, malonate and fumarate (0·05M) and pyrophosphate (0·01M) are quite incapable of giving any protection. All these acids are known to have a high affinity for the enzyme.

(2) In addition to succinate, the enzyme is protected only by sodium dithionite; an aerated solution of sodium dithionite, however, is incapable of giving any protection.

(3) If the enzyme is incubated in the complete absence of air with cyanide and succinate at a concentration sufficient only to give a partial protection, not only the rate of inactivation is reduced, but the inactivation remains incomplete.

Therefore it seems evident that succinate and sodium dithionite protect by keeping the enzyme in the reduced state which is not attacked by cyanide. If succinate is present at a concentration sufficient only to give a partial protection, the enzyme is kept in a partly oxidized and partly reduced state, and only the oxidized form is affected by cyanide.

The failure of Leloir & Dixon (1937) to obtain any inactivation by cyanide was probably due to the spontaneous inactivation of the enzyme, and the residual substrate present in the preparation then available to these workers (Ogston & Green, 1935). The findings of the present investigation are not incompatible with those of Potter (1941) that cyanide does not affect the enzyme responsible for the reduction of cytochrome *c* by succinate. Potter is mainly concerned with the amount of cytochrome *c* reduced by the enzyme system, whereas a partial inactivation of succinic dehydrogenase would affect the rate of cytochrome *c* reduction only.

It is practically certain that the reaction between cyanide and succinic dehydrogenase does not play any part in the mechanism of the cyanide inhibition of respiration since the enzyme is inactivated to an appreciable extent only after being allowed to remain in contact with cyanide in the complete absence of substrate for a long time.

Several suggestions have been made on the question of the chemical nature of succinic dehydrogenase and some of these suggestions will be briefly examined in the light of the present study.

Metal enzyme. Banga & Porges (1938) studied the inhibitory effect of some forty compounds and claimed to have obtained a correlation between the inhibitory and copper-combining powers of these compounds. Contrary to the view held by Leloir & Dixon (1937), Massart, Dufait & van Gremberger (1940) believed that the pyrophosphate inhibition of succinic dehydrogenase was non-competitive. They argued, therefore, that pyrophosphate acted by complex formation with a metal, probably manganese. However, Massart *et al.* (1940) did not produce any convincing evidence to show the pyrophosphate inhibition to be non-competitive. As the pyrophosphate inhibition has now been shown to be competitive, the only evidence in support of the claim of Massart *et al.* is removed. It is difficult to reconcile the view that copper is an integral part of this enzyme with the known strong inhibitory effect of this metal (Hopkins, Morgan & Lutwak-Mann, 1938; Horecker, 1939; Slater, 1949*c*) and the fact that diethyldithiocarbamate does not inhibit in the absence of air (Keilin & Hartree, 1940).

Cytochrome b. It has been pointed out that cytochrome *b* is involved in the anaerobic oxidation of succinate by methylene blue (Slater, 1949*b*). Bach, Dixon & Zerfas (1946) and Ball, Anfinson & Cooper (1947) have made the suggestion that succinic dehydrogenase may be identical with cytochrome *b*. Recently, Pappenheimer & Hendee (1949) examined the succinic dehydrogenase system in *Corynebacterium diphtheriae* in some detail, and expressed the opinion that succinic dehydrogenase is identical with cytochrome *b*. However, these workers produced very little evidence to show the quantitative correlation between succinic dehydrogenase activity and the intensity of the α-band of

ferrocytochrome *b*. As a result of the present work, the following evidence may be summarized in favour of the view that succinic dehydrogenase is not identical with cytochrome *b*.

Spectroscopic observations failed to reveal any parallel relation between the intensity of the α-band of ferrocytochrome *b*, when reduced by succinate, and the succinic dehydrogenase activity. When the succinic dehydrogenase was partially inactivated the rate of reduction of cytochrome *b* was delayed. The intensity of the α-band of ferrocytochrome *b*, when it eventually appeared, was as great as in the untreated enzyme.

When the enzyme was almost completely inactivated (activity less than 2% of the original) an appreciable amount of cytochrome *b* could still be reduced either by dihydrocozymase (Slater, 1950) or by succinate together with a few drops of untreated enzyme.

All cases of cyanide inhibition of haem proteins are reversible, no irreversible inactivation of a haem protein by cyanide being known.

If this view is accepted, the findings of Stoppani (1947, 1949) can also be easily understood. Stoppani found that narcotics inhibited the reduction of methylene blue but not the reduction of ferricyanide by succinate, and a liver preparation could only utilize ferricyanide as the acceptor, although the latter has not been confirmed by Keilin & Hartree (1949). Stoppani's findings can be explained by assuming that ferricyanide is capable of reacting directly with the dehydrogenase, whereas methylene blue can only react with cytochrome *b* (analogous to the coenzyme-linked dehydrogenases and diaphorase, Dixon & Zerfas, 1940) and that narcotics affect the interaction of the dehydrogenase and cytochrome *b*.

It may also be pointed out here that all the experimental facts now available do not exclude the possibilities of (1) a single enzyme molecule with two active centres concerned with succinic dehydrogenase and cytochrome *b* activity respectively, or (2) a separate factor operating between the true dehydrogenase and cytochrome *b*, this factor being affected by cyanide. The first possibility does not appear to be very likely, and unless some evidence is provided it seems unnecessary to postulate a separate factor.

Flavin. Keilin & Hartree (1940) observed two flavin bands at 455 and 495mμ. in their heart-muscle preparation and showed that these bands disappear on reduction with succinate. Axelrod, Potter & Elvehjem (1942) reported that livers of rats fed on a riboflavin-deficient diet were low in succinic oxidase activity. Since all the other known components of the succinic oxidase system are not flavin compounds, it is not improbable that the observed effect should be on the dehydrogenase itself. One may also recall that the 'cytochrome *b*' preparation of Yakushiji & Mori (1937) was supposed to contain a flavin. The only other dehydrogenase which is inactivated by cyanide in this peculiar way is xanthine dehydrogenase with flavin-adenine dinucleotide as its prosthetic group. It must be pointed out, however, that there are significant differences in the cyanide inactivation of xanthine and succinic dehydrogenases and the point of attack of cyanide on either enzyme is not yet clear. Recently, preparations of succinic dehydrogenase in soluble form have been briefly reported (Scott, 1950; Morton, 1950); however, final judgement on this matter must await further information.

SUMMARY

1. Succinic dehydrogenase was slowly but irreversibly inactivated by cyanide. The rate of inactivation depended upon the temperature of incubation and the cyanide concentration. The time required for 50% inactivation was inversely proportional to the cyanide concentration.

2. The rate of inactivation was independent of the pH of incubation from pH 6·5 to 8, indicating that the reaction involved hydrocyanic acid and not cyanide ion.

3. The enzyme could be protected from inactivation by succinate and sodium dithionite but not by malonate (50 mM), fumarate (50 mM), pyrophosphate (10 mM), cystine (2 mM) and cysteine (4 mM). The minimum concentration of succinate required to give complete protection was 2 mM and the minimum concentration to give a measurable protection, 0·02 mM.

4. Evidence has been summarized to show that succinic dehydrogenase, apart from being concerned with the specific activation of succinate, is actually its immediate electron acceptor, and only the oxidized form of the enzyme is susceptible to the attack by cyanide.

5. Additional evidence was obtained showing that the pyrophosphate inhibition of succinic dehydrogenase is competitive in nature.

6. Matching of the intensity of the α-band of reduced cytochrome *b* of cyanide-treated enzyme preparations showed no parallel relation with the succinic dehydrogenase activity of these enzyme preparations. After nearly complete inactivation of the dehydrogenase, cytochrome *b* can still be oxidized and reduced by various means. This evidence, and the fact that all cases of cyanide inhibition of haem proteins are reversible, led to the conclusion that succinic dehydrogenase is not identical with cytochrome *b*.

7. The possible chemical nature of succinic dehydrogenase has been discussed.

I wish to thank Prof. D. Keilin, F.R.S., for his valuable advice and interest in this work.

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Studies on Plant Formic Dehydrogenase

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Thunberg (1921, 1936) first discovered a formic dehydrogenase in the seeds of *Phaseolus vulgaris* (French bean). The same enzyme, obtained from peas by Fodor & Frankenthal (1930), was found to differ from the formic dehydrogenase of bacteria in that it required a coenzyme present in boiled pea juice. Andersson (1934) and Lichtenstein (1936) were able to identify this factor as coenzyme I (Co I). In 1937, Adler & Sreenivasaya, again using peas, studied the formic system in some detail, drawing attention to the strong inhibition obtained with low concentrations of cyanide. The widespread occurrence of the dehydrogenase in plant seeds and variation in the amount of enzyme during the life cycle of the pea has been reported (Davison, 1949*a*). The enzyme is most active in the mature dried seed and diminishes in activity with germination and growth. It increases again in the pod and developing seed.

The work described in this paper has been directed to finding a function for formic dehydrogenase in the metabolism of the plant. The enzyme has been purified 21-fold in 42% yield. Some observations on the properties of the dehydrogenase, including the Co I specificity, the effect of some inhibitors, especially some metal-binding reagents, and experiments on the coupling of the formic system with other dehydrogenases, are reported. Attempts to reverse the oxidation of formate by the dehydrogenase are described. The reduction of certain dyes by the system has been investigated, and attempts have been made to reverse the reaction by poising against one of these dyes (benzyl viologen). Other experiments designed to show such a reversal are those on the effect of carbon dioxide on Co I reduction, and the coupling of the system with the oxidation of triosephosphate. The possible significance of the system in the metabolism of the plant is discussed.

METHODS AND MATERIALS

Pea seeds. Pisum sativum (var. Meteor) were used.

Bean seeds. Phaseolus multiflorus (var. Giant White Czar) were used throughout this work.

Coenzyme I. This was prepared by the method of Williamson & Green (1940), modified by Ochoa (1948). It was 41% pure when estimated by reduction either with lactic dehydrogenase and lactate in the presence of cyanide, or with formic dehydrogenase and formate.

Coenzyme II. This was prepared by the method of Warburg, Christian & Griese (1935), modified by Haas (1947), and found to be 50% pure when estimated by the heart *iso*citric dehydrogenase-aconitase preparation of Adler, Euler, Günther & Plass (1939). The method of estimation, as with Co I, depends on the measurement of reduced coenzyme in an open tube in the Beckman quartz

spectrophotometer at 340 mμ. The value for purity for coenzyme II (Co II) is subject to slight error, since the enzyme preparation contained a fairly active Co II reductase which is slightly autoxidizable.

Heart diaphorase. This was prepared by the method of Straub (1939).

I am grateful to Mr E. J. Morgan of this department for the preparation of the coenzymes and the heart diaphorase.

Estimation of formic dehydrogenase

In place of methylene blue used by previous investigators, thionine was chosen as the reversibly oxidizable dye in most of the estimations, since it was found to be more efficient for plant dehydrogenases. The reduction of the dye was followed in evacuated Thunberg tubes at 38°. The test system was that given in Table 1. Under these experimental conditions the time for sensibly complete reduction of the dye is inversely proportional to the amount of enzyme added. One enzyme unit is defined as the quantity of enzyme reducing 1 μmol. of dye in 1 min.; thus if x μmol. are reduced in t min. the number of enzyme units present is x/t. The activity of the enzyme is expressed as units/mg. N in the preparation, i.e. μmol. thionine/mg. N/min.

Excess heart diaphorase was used because plant Co I diaphorase varied in the different fractions, and there was never enough present to activate the system completely. These observations differ from those of Adler & Sreenivasaya (1937), who found that yellow enzyme caused increased O_2 uptake when the system was tested aerobically, but did not affect the reduction of methylene blue. In a few later experiments a partially purified Co I diaphorase from pea seedlings (Davison, 1950) was used in place of the heart diaphorase.

In many experiments the reaction

$$HCOOH + \tfrac{1}{2}O_2 \rightarrow CO_2 + H_2O$$

has been followed by O_2 uptake in Warburg manometers fitted with flasks carrying two side arms. The incubation temperature was 38° and the test system was that given in Fig. 1. All formate added is completely oxidized, and a theoretical O_2 uptake obtained. Since the purified enzyme will not oxidize other related compounds such as acetic, oxalic, lactic and pyruvic acids, formaldehyde and acetaldehyde, it may possibly prove an effective tool for the estimation of formate.

To absorb CO_2 produced in the reaction, NaOH was placed in the centre well, and, in experiments using cyanide, this was replaced by a cyanide-alkali mixture (Krebs, 1935).

In the earlier stages of purification the cloudiness of the preparations precluded the use of the Beckman spectrophotometer in following the reduction of Co I, although this method was used with later clear preparations.

Purification of formic dehydrogenase. Preliminary experiments using pea and white bean seeds showed that extraction of a dry powder obtained by grinding the seeds finely in a coffee grinder and sieving through bolting silk (50–60 meshes/in.) was more effective than the use of the Waring blender in homogenizing soaked seeds. Drying the seeds for 3 hr. at 40° did not affect the activity of the enzyme and facilitated grinding.

Alkaline phosphate was used by Adler & Sreenivasaya (1937) in preparing formic dehydrogenase. Although this was not the most efficient extractant when compared with water and phosphate buffers of various pH values, it was used in the large-scale preparations because extracts made with 0·1 M-Na_2HPO_4 were more stable to dialysis and storage and it also provided the necessary buffering action when $(NH_4)_2SO_4$ was used for subsequent precipitation. With water extracts it was found that treatment with 80% $(NH_4)_2SO_4$ followed by dialysis against distilled water destroyed formic dehydrogenase activity, completely in pea preparations and partially in bean preparations. This inactivation could be partly prevented by dialysis against running tap water, which had some buffering action, and completely prevented by dialysis against 0·01 M-Na_2HPO_4. The same pH inactivation effect was encountered in experiments aiming to clarify the cloudy water extract by dialysis against water in which various amounts of CO_2 were dissolved. Dialysis for 1 or 2 hr. against distilled water 1/10 saturated with CO_2 did not affect the enzyme. Dialysis for 4 hr. decreased the activity by 50%, and after 8 hr. no activity remained.

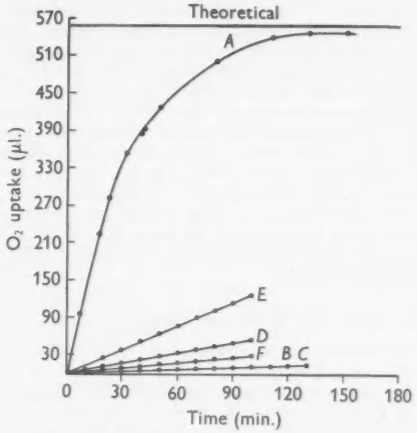

Fig. 1. Aerobic estimation of formic dehydrogenase. The complete test system: 1·8 ml. enzyme preparation (a freeze-dried preparation from fraction (8), 15 mg./ml.); 0·6 ml. 0·3 M-phosphate buffer, pH 7·0; 0·3 ml. Co I, 5 mg./ml.; 0·1 ml. heart diaphorase, containing 1 μg. bound riboflavin; 0·1 ml. 0·005 M-methylene blue; 0·1 ml. 0·5 M-sodium formate (≡560 μl. O_2) in the side bulb; distilled water to a final volume of 3·0 ml. In the centre well, 0·2 ml. 20% (w/v) NaOH. Formate tipped in from side bulb to start reaction. Curve *A*, complete system; curve *B*, no formate; curve *C*, no coenzyme; curve *D*, no heart diaphorase; curve *E*. no methylene blue; curve *F*, no diaphorase or methylene blue.

For large-scale purification beans were chosen because here the initial blank was smaller, the formic enzyme was more stable to acid conditions, and there was less loss on storing extracts. The enzyme was purified as follows.

Sifted bean meal (150 g.) was extracted with 450 ml. 0·1 M-Na_2HPO_4 for 1 hr. at room temperature with occasional stirring. The homogenate was squeezed through muslin, centrifuged for 15 min. at 2800 rev./min., and the supernatant (1) treated with solid $(NH_4)_2SO_4$ to 0·8 saturation. The precipitate was filtered off on kieselguhr, and the cake rubbed up with 75 ml. 0·03 M-Na_2HPO_4. This suspension

was dialysed against running tap water overnight. The use of alkaline phosphate in taking up the precipitate, and running water for dialysis was adopted instead of distilled water since use of the latter decreased the yield, due to acid conditions. The dialysate was spun for 30 min. at 3600 rev./min. and a large amount of inert protein removed. The supernatant (2), about 200 ml., was used for fractionation with calcium phosphate gel (30 mg. dry wt./ml.). Calcium phosphate gel (20 ml./100 ml. supernatant) was added to fraction (2), the suspension mixed and allowed to stand at room temperature for 30 min. The gel was spun off and the supernatant (3) treated again with 20 ml. calcium phosphate/100 ml. supernatant. The supernatant (4) from this adsorption was then treated with 30 ml. calcium phosphate/100 ml. The supernatant (5), after spinning, was treated a second time with 30 ml. calcium phosphate/100 ml. supernatant. Supernatant (6) from this adsorption was then treated with 40 ml. calcium phosphate/100 ml., the enzyme being brought down on the gel at this stage. The supernatant was discarded, and the gel eluted with three successive lots of 25 ml. 0·1 M-Na_2HPO_4 for 15 min. each time. This preparation (7) was neutralized to pH 7·0 with 0·3 M-KH_2PO_4 and dialysed against distilled water at 4° overnight. The dialysed preparation (8) may be stored at −10° for several weeks without loss of activity, or, alternatively, freeze-dried and the dry powder stored. Preparation (7), after neutralizing, may also be stored as a dry powder, but the enzyme does not keep well stored in solution at −10° in the presence of phosphate. The activity and yield at the various stages of purification are given in Table 1. In the final preparation (8) the enzyme was purified 21-fold and a 42% yield obtained.

Table 1. *Purification of formic dehydrogenase*

(Test system: in the Thunberg tube: 1·0 ml. preparation; 0·3 ml. 0·3 M-phosphate buffer, pH 6·0; 0·3 ml. 0·5 M-sodium formate; 0·1 ml. heart-diaphorase preparation, containing 1·2 μg. riboflavin. In the hollow stopper: 0·3 ml. Co I, 5 mg./ml.; 0·4 ml. 0·005 M-thionine. The tubes were incubated for 5 min. at 38° before the Co I and dye were tipped in. Reduction times were between 1 and 4 min. for the series. Fraction (1) is corrected for the blank obtained with this preparation (decolorization times 1·5 and 30 min. in the presence and absence of formate). After this fraction there was no appreciable blank. Overall purification 21-fold; overall yield 42%.)

Fraction no.	Activity (μmol./min./mg. N)	Total enzyme units
1	0·0642	275
2	0·1451	192
3	0·188	186
4	0·266	184
5	0·3605	173
6	0·676	165
7	1·065	151
8	1·347	115

RESULTS

Coenzyme specificity

In experiments on the coenzyme specificity of formic dehydrogenase, Adler & Sreenivasaya (1937) used a preparation of Co II contaminated with Co I, but since the activity of this preparation could be accounted for entirely by its Co I content, they concluded that the enzyme was specific for Co I. This has been confirmed using fraction (8), and a Co II preparation free from Co I. Co II, in four times the concentration of Co I used in Table 1, was completely ineffective in the reduction of thionine, and no appreciable reduction of Co II could be observed in the Beckman spectrophotometer at 300 mμ. in the presence of the enzyme and formate. Mathews & Vennesland (1950) have also confirmed this specificity and report that it is possible to detect as little as 0·01 mg. Co I in the presence of 1·0 mg. Co II, making the enzyme a useful tool for the detection of Co I.

Effect of metal ions

In view of the cyanide sensitivity of formic dehydrogenase, the effect of various metal ions at a concentration of 1×10^{-3} M was tried on fraction (8) in Thunberg experiments. Fe^{++}, Mg^{++} and Mn^{++} had no effect; Fe^{+++} and Cu^{++} were strongly inhibitory, reduction times of >120 min. being obtained, in comparison with 2 min. in the absence of metal additions.

Other inhibitors

Although Fodor, Frankenthal & Kuk (1930) did not obtain cyanide inhibition of formic dehydrogenase using methylene blue as hydrogen acceptor, Adler & Sreenivasaya (1937) found that both methylene blue reduction and oxygen uptake by the formic system are inhibited by 10^{-3} M-cyanide. The results of Adler & Sreenivasaya have been confirmed and $1·3 \times 10^{-3}$ M-cyanide was found to inhibit formic dehydrogenase completely with both methylene blue and oxygen as final hydrogen acceptors. Mathews & Vennesland (1950) also report that the reduction of Co I in their spectrophotometric test was inhibited more than 95% by 10^{-3} M-cyanide.

Besides cyanide, other metal-combining compounds have been tried on the purified formic dehydrogenase (Table 2). Azide, in a concentration of $1·3 \times 10^{-3}$ M, completely inhibits the oxidation of formate, both aerobically and anaerobically. The cyanide and azide inhibition was found to be completely removed by dialysis. Other heavy-metal combining substances, such as hydroxylamine, 8-hydroxyquinoline and *o*-phenanthroline had no effect. The effect of carbon monoxide was tried and a mixture of 4 parts carbon monoxide to 1 part oxygen showed a 15% inhibition of the oxygen uptake by the enzyme system, but this inhibition was diminished only very slightly by light. Diethyldithiocarbamate ($0·5 \times 10^{-2}$ M) was found to activate the enzyme slightly both aerobically (18% activation) and anaerobically (12%). 2:3-Dimercaptopropanol (BAL) had no effect aerobically at a concentration

of 1×10^{-3}M. Anaerobically, difficulty was experienced with the non-enzymic reduction of dyes which are rapidly decolorized by the strongly reducing BAL. However, on a percentage blank basis, the substance did not have any effect on the enzyme.

Table 2. *Effect of inhibitors of formic dehydrogenase*

(Contents of manometer flasks: 1·0 ml. enzyme fraction (2); 0·6 ml. 0·3M-phosphate buffer, pH 7·0; 0·3 ml. 0·5M-sodium formate; 0·1 ml. heart-diaphorase preparation containing 1·2 μg. bound riboflavin; 0·3 ml. CoI, 5 mg./ml.; 0·1 ml. 0·005M-methylene blue; 0·3 ml. water or inhibitor. In the centre well, 0·2 ml. 20 % alkali or alkali-cyanide mixture (Krebs, 1935). The inhibitor was incubated with the enzyme preparation at 38° for 10 min. before tipping in the formate from the side bulb.)

Final concentration of the inhibitor	O_2 uptake in 60 min. (μl.)
No inhibitor	134
$0{\cdot}5 \times 10^{-2}$M-Diethyldithiocarbamate	159
Carbon monoxide (80 %) in the gas phase (dark)	109
Carbon monoxide (80 %) in the gas phase (light)	114
1×10^{-3}M-BAL	139
$1{\cdot}3 \times 10^{-3}$M-Azide	0
$1{\cdot}3 \times 10^{-3}$M-Cyanide	13

Malonate in concentrations of $1{\cdot}3 \times 10^{-3}$M did not inhibit the aerobic system; and 4×10^{-2}M-semicarbazide had no effect. Iodoacetate, even up to a concentration of 0·1M, had no effect, but two other —SH reagents were inhibitory. *p*-Chloromercuribenzoate was found to inhibit the anaerobic system completely at 1×10^{-3}M, and iodosobenzoate inhibited about 60 % at this concentration.

Attempted reversal of the oxidation of formate

In Fig. 2 the positions of the half-reduced oxidation-reduction systems, carbon dioxide-formate, CoI-reduced CoI, and benzyl viologen-reduced benzyl viologen, have been plotted on an *r*H scale. Calculated from the distance between the coenzyme and formate curves the equilibrium constant of the reaction between these two systems, at pH 7·0 and 30°, is approximately $1{\cdot}1 \times 10^4$. Attempts to reverse this reaction were made in three types of experiment.

(1) *Poising the system against a dye of low E'_0, and the effect of carbon dioxide on the percentage reduction of the dye*

Several dyes were tried to see if the formic system could be poised against one of them (Table 3). Neutral red ($E'_0 = -0{\cdot}325$ V.) was not reduced at all. Janus green ($E'_0 = -0{\cdot}255$ V.) was reduced slowly to the red amino compound, but not further to the leuco form. Although the reduction was rather slow with benzyl viologen ($E'_0 = -0{\cdot}359$ V.) and methyl viologen ($E'_0 = -0{\cdot}446$ V.), both these dyes were found to be satisfactory in that the final colour was less than the full reduction colour.

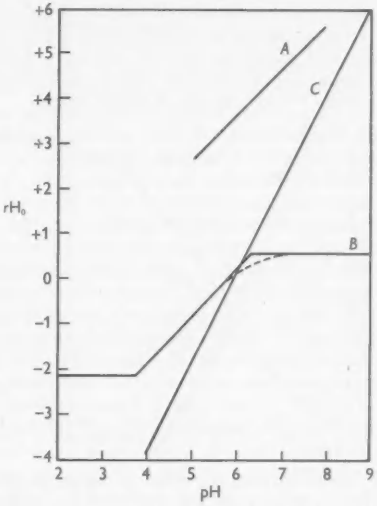

Fig. 2. *r*H curves for half-reduced oxidation-reduction systems. Curve *A*, Co I; data from Borsook (1940). Curve *B*, formate-bicarbonate-carbonate; from the thermochemical data on free energies (Woods, 1936). Curve *C*, benzyl viologen; data from Michaelis & Hill (1933).

Table 3. *Reduction of various dyes by the formic system*

(Thunberg contents: 1·0 ml. freeze-dried preparation from fraction (8), 10 mg./ml.; 0·6 ml. 0·2M-phosphate buffer, pH 7·0; 0·3 ml. 0·5M-sodium formate; 0·1 ml. heart diaphorase (1·2 μg. bound riboflavin), or 0·1 ml. water. In the hollow stopper: 0·3 ml. CoI, 5 mg./ml.; 0·4 ml. 0·0025M-dye. Incubation at 37°.)

Dye	Reduction time (min.) With flavoprotein	Reduction time (min.) Without flavoprotein
Thionine	0·58	1
Methylene blue	3	4·5
Benzyl viologen	15	45
Methyl viologen	25	50
Benzyl viologen + thionine	0·58	1
Neutral red	>180	>180
Janus green	10	90

That benzyl viologen is not inhibitory is seen in the experiment where it is used in addition to thionine, when the thionine was decolorized before the benzyl viologen started to show its blue reduction colour. The possibility that the coenzyme was being destroyed during the longer times with benzyl viologen was tested by reopening the tubes to

re-oxidize the dye. On re-evacuating, the same colour again developed, and at approximately the same rate.

Fig. 3 gives the results of experiments on the percentage reduction of benzyl viologen, with increasing pH, in the presence of equimolecular amounts of formate and dissolved carbon dioxide. A curve representing the theoretical reduction, calculated from curves *B* and *C* of Fig. 2, is also given.

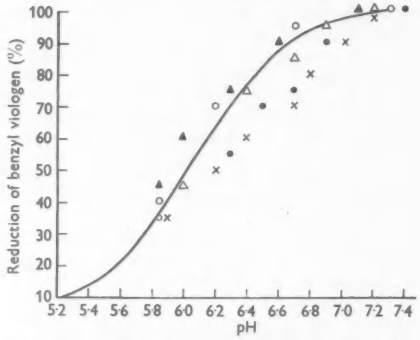

Fig. 3. Percentage reduction of benzyl viologen at different pH values. The whole line represents the theoretical percentage reduction of the dye as calculated from the curves of Fig. 2. The points marked are those obtained in five experimental runs. Thunberg contents: 1·0 ml. fraction (8); 2·0 ml. $NaHCO_3$ (molarity depending on the pH required and calculated from the Henderson-Hasselbach equation); 0·3 ml. 0·3 M-sodium formate; 0·1 ml. heart diaphorase (1·2 μg. bound riboflavin); 0·2 ml. Co I, 5 mg./ml.; 0·4 ml. 0·0025 M-benzyl viologen. Temperature of incubation, 30°. The tubes were evacuated and gassed with CO_2, giving a concentration of dissolved CO_2 of 0·02 M. The gas was freed from O_2 by passing over heated copper, then through a flask containing freshly cut yellow phosphorus. A small piece of yellow phosphorus was placed in the hollow stopper of each tube, and the dye added to the other components just before evacuation. Standards for comparison of the colour were made by reducing with $Na_2S_2O_4$ known concentrations of benzyl viologen corresponding to a range of percentage reduction colours.

When these experiments were repeated in the absence of carbon dioxide, the percentage reduction of the dye was increased by about 20%. These results, however, were within the limits of experimental error, as seen from the wide scatter of points in Fig. 3. The presence of adenosinetriphosphate (ATP) had no effect on the percentage reduction of the dye, either in the presence or absence of carbon dioxide.

(2) *Effect of carbon dioxide on the percentage reduction of Co I*

A Thunberg tube fitted with an optical cell at the base was used for these experiments, in which the reduced Co I was measured by its absorption at 340 mμ. in the Beckman spectrophotometer. Adler & Sreenivasaya (1937) found that 85% of added Co I was reduced by the formic system, but this was assuming 100% purity for the Co I. Using fraction (8), either in the above apparatus or in an open tube, Co I added was completely reduced in the presence of formate. When bicarbonate buffer was used and the Thunberg tube gassed with carbon dioxide, no decrease in the reduction of Co I could be observed. In the absence of formate there was no re-oxidation of added reduced Co I by the formic dehydrogenase and carbon dioxide.

(3) *Attempted reversal by coupling with the oxidation of triosephosphate*

Attempts were made to reverse the formate oxidation by coupling carbon dioxide reduction with a reaction producing reduced Co I, and high-energy phosphate in the form of ATP. Such a reaction is the oxidation of triosephosphate to phosphoglyceric acid. Since inorganic phosphate is taken up in this oxidation and passed on to adenosinediphosphate (ADP) (which in turn, in the presence of added yeast hexokinase and glucose, will hand it on to form glucose-6-phosphate), if there is any coupling with a system which will re-oxidize the reduced Co I, this will be shown by an uptake of inorganic phosphate. Hexosediphosphate was used as substrate in trying to show this, in the presence of bean extract, Mg^{++} ions, ATP, Co I, yeast hexokinase, glucose and bicarbonate buffer, the manometers being gassed with carbon dioxide. There was no significant decrease in inorganic phosphate after 1 hr. incubation, i.e. the carbon dioxide-formate system was not re-oxidizing the reduced Co I. That all the enzymes necessary for the production of triosephosphate from hexosediphosphate and for the subsequent oxidation of triosephosphate were present in the crude extract was seen when acetaldehyde and alcohol dehydrogenase (also present) were used as the re-oxidizing system. Here there was a 20% reduction of the inorganic phosphate in 30 min.

Carrier-linked dismutations

It has been shown that the oxidation of formate can be linked in crude pea extract with the reduction of acetaldehyde and of fumarate, through the mediation of Co I, or Co I plus some other carrier (Davison, 1949*b*). Another dismutation which can easily be demonstrated is that between formate and formic dehydrogenase as the negative system, and α-ketoglutarate, ammonia and glutamic dehydrogenase as the positive system. This reaction goes particularly well because the formic system is strongly reducing, and the equilibrium of the glutamic system is far towards the side of glutamate formation (Euler, Adler, Günther & Das, 1938).

Sifted bean meal (10 g.) was stirred up with 20 ml. 0·2M-phosphate buffer, pH 7·4, and left to stand 2 hr. The homogenate was centrifuged and the supernatant used in the experiment described in Table 4.

Table 4. *Coenzyme-linked dismutation between formic and glutamic dehydrogenases*

(Contents of manometer flasks: 1·0 ml. bean supernatant; 0·3 ml. 0·09M-$NaHCO_3$ (to give a pH of 7·2 when gassed with 5% CO_2 in N_2 at 38°); 0·4 ml. CoI, 5 mg./ml.; 0·2 ml. α-ketoglutarate; 0·8 ml. 0·3M-NH_4Cl; 0·3 ml. 0·4M-sodium formate (in the side bulb); water to 3·0 ml.)

Omissions from flask	CO_2 evolved in 60 min. (μl.)
No omissions	272
Ammonia	38
α-Ketoglutarate	16
Coenzyme I	22
Ammonia and α-ketoglutarate	12

The increase in carbon dioxide output with the complete system is due to increased oxidation of formate in the presence of a system capable of re-oxidizing reduced CoI.

DISCUSSION

Three distinct enzyme systems capable of oxidizing formic acid have now been described. The plant formic dehydrogenase is characterized by its dependence on CoI, and is the only coenzyme-linked dehydrogenase found to be inhibited by cyanide. The formic dehydrogenase of *Escherichia coli* (Gale, 1939) does not need CoI, and has been shown to be cytochrome-linked. Here, cyanide does not inhibit the reduction of methylene blue, but strongly inhibits the aerobic oxidation of formate because of its effect on the cytochrome system (Cook, Haldane & Mapson, 1931).

In animals, Wishart (1923) showed that extracts of rabbit liver will catalyse the reduction of methylene blue by formate. Recently, Mathews & Vennesland (1950) have obtained soluble preparations from rat liver and kidney which would oxidize formate. The oxidation was inhibited about 85% by 10^{-3}M-cyanide. It is not clear what the immediate hydrogen acceptor is here; the system was not activated by CoI, but differed from the bacterial enzyme in that either ATP or adenylic acid were necessary for oxygen uptake.

Mathews & Vennesland report that ATP has no effect on the activity of the plant formic dehydrogenase, and this has been confirmed by anaerobic experiments using benzyl viologen. To investigate the possibility of some other mechanism for the formation of formyl phosphate in plants, extracts of bean have been incubated with formate, ATP, Mg^{++} ions, CoI and hydroxylamine. If any formyl phosphate were formed this would be trapped by the hydroxylamine. After 30 min. incubation the solutions were examined for hydroxamic acid by the method of Lipmann & Tuttle (1945). None was present.

In considering the possible significance of formic dehydrogenase in higher plants, if the enzyme is looked upon as a carboxylase, the suggestion that it may be involved in carbon dioxide fixation at once arises. Two pathways involving carboxylases are known to occur in higher plants for the heterotrophic fixation of carbon dioxide. Vennesland & Felsher (1946), Vennesland (1949) and Vennesland, Gollub & Speck (1949), showed that the reversible breakdown of oxaloacetate to pyruvate and carbon dioxide occurs in a wide variety of seeds, seedlings, leaves and roots. The carbon dioxide fixation reaction required the presence of ATP. The oxaloacetic carboxylase is closely associated with a malic dehydrogenase, and, in the presence of reduced CoII, the reduction of oxaloacetate to malate takes place. In parsley roots, carbon dioxide fixation has been shown to occur by a reversal of the oxidative decarboxylation of *iso*citrate (Ceithaml & Vennesland, 1949). With a preparation from this source oxalosuccinate is formed from α-ketoglutarate and carbon dioxide, in the presence of cobalt or manganese ions. If reduced CoII is added to this system, *iso*citrate is formed from the oxalosuccinic acid.

The possibility of a third mechanism for carbon dioxide fixation in plants was suggested by the work of Woods (1936) with *Escherichia coli*, in which formic acid is produced from carbon dioxide and hydrogen. However, it was found that the participation of CoI in the oxidation of formate by plants changed the equilibrium constant from 3·81 in the case of the bacterial reaction to $1{\cdot}1 \times 10^4$ when the plant system was used. It has not been possible to obtain the reduction of carbon dioxide to formate by means of the plant formic dehydrogenase. No evidence was obtained that the formate carbon dioxide system can be reversed by the effect of carbon dioxide on the percentage reduction of benzyl viologen, against which the system may be poised. The presence of ATP had no effect on the reduction of the dye either in the presence or absence of carbon dioxide. Nor did coupling with the oxidation of triosephosphate push the reaction in the direction of carbon dioxide fixation. Possibly some physiological reversal does occur in the intact seed, but, if so, it has not been possible to demonstrate this *in vitro*. Since the completion of this work, Mathews & Vennesland (1950) have reported independently that they have not been able to obtain the reduction of carbon dioxide in the presence of a partly purified formic dehydrogenase from pea seeds, nor have isotope experiments of the same authors indicated any significant exchange of labelled carbon between $^{14}CO_2$ and sodium formate in the presence of the enzyme.

Data indicating a much more likely role for formic dehydrogenase are those presented on the use of the enzyme in forming reduced CoI, which can then react with the corresponding oxidized compounds to form ethanol, succinate and glutamate in the presence of their respective dehydrogenases, and, in the case of succinate, some other carrier. Such anaerobic dismutations have an obvious significance in the respiration of large seeds. The ease with which the glutamic reaction goes, especially, and the importance of glutamic acid in the seed, points to a possible role for the strongly reducing formic system in the metabolism of the plant.

Tied up with this is the question of the origin of formate in plants. Formate is said to arise, together with ethanol and acetic acid, during the anaerobic respiration of potatoes and seeds (Stoklasa, Ernest & Chocenský, 1907). Stoklasa & Ernest (1908) found that, when the oxygen supply was limited, formic and acetic acids were excreted from the roots of buckwheat and barley, and formic acid from the roots of oats and maize. Formate is known to arise by the oxidation of glycollic acid in leaves (Clagett, Tolbert & Burris, 1949). However, in the present work, glycollic oxidase has not been found in pea seeds, and in tobacco leaves containing a very active glycollic oxidase there was no formic dehydrogenase. Of some possible precursors of formate, pyruvate is completely decarboxylated by pea and bean extracts; acetate, formaldehyde and glycine do not support oxygen uptake in the presence of CoI, and therefore it is not likely that formate is being produced from these compounds.

In animal tissues formate has been shown to give rise to labile methyl groups (Sakami & Welch, 1950). Also formate, or a one-carbon intermediate of its metabolism, was shown by Sakami (1950) to be formed from acetone by *in vivo* isotope experiments with rats. Formic acid, together with glutamic acid and ammonia, are claimed to be the products of the breakdown of histidine by liver histidase (Walker & Schmidt, 1944). However, none of these reactions has yet been investigated in plants, so that the origin of formate and the role of its dehydrogenase in seeds remain open questions.

SUMMARY

1. A method of purification for formic dehydrogenase from bean seeds is described.

2. The coenzyme I specificity of the enzyme has been confirmed.

3. The effects of various inhibitors on the enzyme have been examined.

4. No reduction of carbon dioxide to formate by means of this enzyme has been obtained.

5. The coenzyme-linked reaction between formate oxidation and the reductive amination of α-ketoglutarate has been shown to occur rapidly in extracts of bean seed.

I am most grateful to Dr R. Hill, F.R.S., for his advice and encouragement, to Dr M. Dixon, F.R.S., and members of the Enzyme Unit of this department for their help and interest in the work, and to the Commissioners for the Exhibition of 1851 for a Scholarship.

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The Colorimetric Determination of Lactic Acid in Silage

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The fundamental reaction which takes place in the process of ensilage is the conversion of a proportion of the soluble carbohydrates present to lactic acid. This reaction is of such importance that when material of high protein content is being ensiled, it is desirable to add molasses to provide a readily available substrate for the lactobacilli which occur along with coliform organisms in the original grass crop. When lactic acid is formed in suitable amount it acts as a preservative for the ensiled material and serves, on account of the lowering of pH which accompanies its formation, to inhibit the activity of coliform bacteria which produce undesirable by-products.

Despite the importance of this reaction it has never been fully investigated, partly on account of the pre-occupation of many investigators with the question of quality in silage, for the determination of which the pH is a reasonable guide, and partly because the methods used for its estimation are tedious and in some cases inaccurate. For example, the method of Foreman (1920) depends upon a difference calculation which involves the experimental error of the other acid determinations, and gives a result which represents the total non-volatile acid content, of which but a part is lactic acid, a fact which has been stressed by Watson & Ferguson (1937) in their study of the method. The method of Karström & Virtanen (1937), modified from the original techniques of Hirsch-Kauffmann (1924) and Lieb & Zacherl (1932), involves the use of large amounts of material and complicated procedures for the elimination of proteins and carbohydrates.

Miller & Muntz (1938) developed a method, later modified by Barker & Summerson (1941), for the colorimetric estimation of lactic acid which depends upon the fact that lactic acid is oxidized by sulphuric acid to acetaldehyde which gives a purple-red colour with *p*-hydroxydiphenyl in the presence of metallic ions, of which copper has been found to be the most suitable (Barker & Summerson, 1941). The reaction is similar to that of Mendel & Goldscheider (1925) who, however, used *o*-dimethoxybenzene as the colour-producing reagent.

Davidson (1949) has used the Barker & Summerson technique for the determination of lactic acid in milk and milk products, demonstrating its applicability to amounts of 20–400 mg. lactic acid. In the case of these latter, and blood or minced tissue, steps have to be taken to remove the protein matter involved. Silage, however, may be looked upon as solid matter covered with, or impregnated with, lactic acid to a greater or lesser extent and the amounts of protein extractable by water from silage are relatively small. Of the 5 g. portions of fresh silage which are used in the method of analysis described below, less than 0·4 g. is protein and of this only a part is soluble in water. This small proportion may be removed from the prepared extract at the same time as the soluble carbohydrates.

The technique of Barker & Summerson, as used for the determination of lactic acid in silage, is described below.

REAGENTS

Standard lactic acid solution. Pure zinc lactate (0·0135 g.) is dissolved in 10 ml. of distilled water contained in a 1 l. standard flask. Conc. H_2SO_4 (0·5 ml.) is added and the volume made up to the mark with distilled water. This solution is stable but should be kept in a refrigerator.

p-Hydroxydiphenyl reagent. *p*-Hydroxydiphenyl (1·5 g.) is dissolved in 10 ml. 5% (w/v) aqueous NaOH and made up to a litre with water. This reagent is also stable but should be stored in an amber bottle.

Calcium hydroxide. The brand used is Judex.

Copper sulphate solutions. Two $CuSO_4$ solutions are required, one 0·80 M and the other 0·16 M. These are prepared from A.R. $CuSO_4.5H_2O$.

Concentrated sulphuric acid. It has been shown by Russell (1944) that nitrates and nitrites interfere with the reaction, and for this reason the N-free H_2SO_4 essential for Kjeldahl determinations is used.

METHOD

Of the representative sample of silage (about 3 kg.) brought to the laboratory for examination, about 100 g. taken from the centre of the mass is finely chopped and treated after the fashion described below. A further 100 g. portion is mixed with 100 ml. of distilled water in a beaker and set aside for 24 hr. pending a pH determination on the extract. A dry-matter determination is performed on approximately 1 kg. of the material by drying this at 80° for 24 hr.

Of the chopped material two 5 g. portions are placed in two 250 ml. beakers and 100 ml. of boiling distilled water added and the whole allowed to cool

for 5 min. Each preparation is treated in the following way. The mixture is stirred mechanically very vigorously for 5 min., during which time about 0·5 g. of solid $Ca(OH)_2$ is added to assist the subsequent filtration. The product is filtered through a hard filter paper and the first 20 ml. of filtrate are discarded. Of the filtrate 2 ml. are taken and diluted to 10 ml. with distilled water, and of this solution 1 ml. is placed in a small glass-stoppered bottle to which are added, in the following order, 8 ml. of distilled water from a burette, 1 ml. of 0·80 M-$CuSO_4$ and 1 g. of $Ca(OH)_2$. A standard for comparison is prepared by using 5 ml. of standard lactic acid solution and making this up as described for the test portion, using only 4 ml. of distilled water. Similarly, a blank is prepared by using 1 ml. of water instead of the unknown. The four bottles are then stoppered and shaken for 20 min. in a small mechanical shaker, after which time the contents are filtered through small filter papers discarding the first 2 ml. of filtrate in each case. The remainder of each filtrate is collected in small test tubes. Each filtrate is treated as follows: The filtrate (1 ml.) is placed in a Pyrex tube (200 × 30 mm.) and mixed with 0·05 ml. of 0·16 M-$CuSO_4$ and 6 ml. of N_2-free H_2SO_4 added from a burette with grease-free tap. The tubes are allowed to stand in boiling water for 5 min. and are then cooled by standing in a stream of running water. To each tube is added 0·1 ml. of the *p*-hydroxydiphenyl reagent and the contents shaken laterally to ensure even distribution of the insoluble reagent. The tubes are then placed in a water bath for 30 min. at 30–33°, being shaken at 10 min. intervals. Finally, the tubes and their contents are heated for 2 min. in a bath of boiling water and cooled to room temperature. Readings of the colour developments are made on an EEL (Evans Electroselenium Ltd., Harlow, Essex) photoelectric colorimeter using the standard green filter supplied by the makers and water as a zero. As the 1 ml. copper-containing portion of standard lactic acid contains 0·005 mg./1 ml. portion used, the concentration of lactic acid in the unknown is given by the formula:

$$\% \text{ Lactic acid in fresh silage} = \frac{\text{Reading for unknown } (-\text{blank})}{\text{Reading for standard } (-\text{blank})} \times 0{\cdot}5,$$

and for the amount of lactic acid based on the dry matter (DM) content of the silage:

$$\% \text{ Lactic acid} = \frac{\text{Reading for unknown } (-\text{blank})}{\text{Reading for standard } (-\text{blank})} \times \frac{50}{\% \text{ DM content}}.$$

The results of a control experiment to test the accuracy of the method are indicated in Fig. 1, while recovery of lactic acid from mixtures of known standard and unknown varies from 96 to 102 %, and the only precaution required is absolute cleanliness as, owing to the sensitiveness of the reaction, impurities which give rise to lactic acid on heating with H_2SO_4 nullify the results. All glassware used is successively washed with dil. HCl, warm water with a little detergent, cold water and finally dried in an oven.

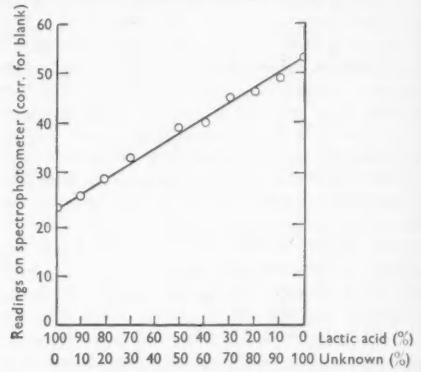

Fig. 1. Spectrophotometer readings for mixtures of standard lactic acid solution and silage extract as prepared for analysis in percentages shown (by vol.). For details of procedure see Text.

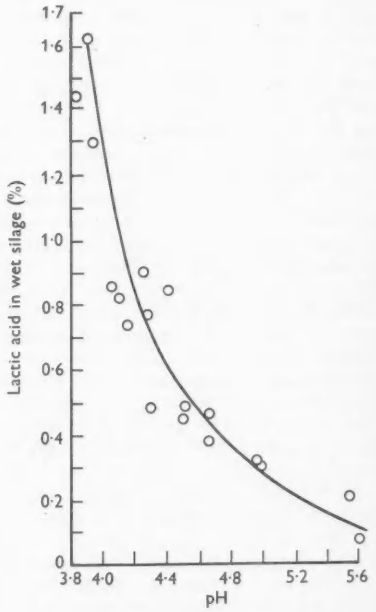

Fig. 2. Relation of lactic acid content of wet silage to pH.

RESULTS

The silage specimens on which data are reported were from grass and grass-clover mixtures ensiled during the summer of 1950 on various farms in the north-east of Scotland, and were taken at random from specimens being analysed here.

It is seen from Fig. 2 that the lactic acid content of silage falls rapidly with rise of pH. This is in accordance with the generally held view that in silage of pH greater than 4·2 the amounts of lactic acid are small. It has not been definitely shown, however, whether or not lactic acid is formed in all silage but is subsequently destroyed in silage of high pH. An investigation into this problem is the subject of further work.

SUMMARY

The use of the procedure of Barker & Summerson (1941) for determining lactic acid is described in its relation to the estimation of this acid in silage.

It is desired to thank Mr John Baxter for his assistance in the technical side of the present work.

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Transfructosidation in Extracts of the Tubers of *Helianthus tuberosus* L.

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In the artichoke tuber there is present a mixture of carbohydrates, of which sucrose is the component with smallest molecular weight, and inulin probably the largest (Bacon & Edelman, 1951). Since the only enzyme system which is known to produce a fructose polysaccharide *in vitro* (levansucrase; Hestrin, Avineri-Shapiro & Aschner, 1943) does so from sucrose, it seemed worth while to investigate the possibility that inulin was formed from sucrose, and with this aim in view the action of tuber extracts on sucrose was studied.

The results presented in this paper show that a reaction takes place in tuber extracts by which oligosaccharides are synthesized from sucrose at the expense of fructose residues from inulin and related polysaccharides. The name 'transfructosidation' is proposed for this type of reaction. A preliminary account was given to the Biochemical Society at Edinburgh on 21 July 1950 (Edelman & Bacon, 1950).

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METHODS

The methods were those described by Bacon & Edelman (1951) and Edelman & Bacon (1951).

Dandelion inulin (Thomas Kerfoot and Co. Ltd., Vale of Bardsley, Ashton-under-Lyne) was purified as described by Edelman & Bacon (1951). Sucrose was recrystallized one or more times from 95% aqueous ethanol. Both carbohydrates were shown by paper partition chromatography to be free from other oligosaccharides and free hexoses.

All incubations were carried out in the presence of $CHCl_3$ unless otherwise stated.

RESULTS

Initial investigation

Qualitative experiments. It was assumed that any synthetic activity in the presence of sucrose would lead first to the production of oligosaccharides behaving, on the paper partition chromatogram, like spots 2, 3 and so on (Bacon & Edelman, 1951). Since these substances were already present in the tubers, dialysis was carried out to remove them.

Mash, prepared in November 1949, was dialysed against running cold water for 50 hr., the dialysis sac being periodically agitated by a rocking movement. Two 25 ml. samples of the dialysed material were adjusted to pH values of 5·0 and about 6·5 with 1 ml. M-sodium acetate buffer and drops of 0·2N-sodium hydroxide respectively. Samples (10 ml.) of these preparations were each incubated at 28° with 1 ml. 50% sucrose. Other samples were incubated without added sucrose. Chromatograms of 20 and 45 hr. incubation samples containing added sucrose showed, at both pH values, a spot in position 2 of the artichoke carbohydrate series. The higher pH value gave a rather more pronounced effect as judged qualitatively. Spot 2 did not appear in the controls. Although free glucose and fructose appeared in rather greater amount in the samples incubated with sucrose than in the controls, only small amounts of these sugars were formed. Further experiments, similar to the one described, confirmed that spot 2 appeared in the presence of sucrose, but not in its absence, and showed that it was formed in greater amount at pH values 5·8 and 6·4 than at 4·2 and 7·2. Boiled extract in the presence of sucrose did not give the reaction. Some of the incubated samples in these experiments contained only traces of monosaccharide, although considerable amounts of spot 2 had been formed (see Fig. 1).

Quantitative experiments. The effect was studied in undialysed mash by developing the use of quantitative paper partition chromatography to follow changes in the amounts of the oligosaccharide components. Two samples of 25 g. mash were incubated at 28° with 2·5 ml. 33% sucrose and 2·5 ml. water respectively. No pH adjustment was made. Samples of the liquid strained from the incubated mash through gauze were chromatographed in butanol-acetic acid and the ketose content of the spots estimated. Results are given in Table 1.

A micrometer syringe was not used in applying the 5 μl. samples of liquid to the filter paper in this experiment; a 0·1 ml. graduated pipette was used instead, with consequent loss in accuracy. In spite of this, however, the table shows that there were no major changes in any of the control incubations except in spot *F* (free fructose) and spots 5 to *n*. Spots 2 and 3 in the sample incubated with sucrose, however, showed a very marked increase, and there was a smaller but probably significant increase in spot 4. Spot 1 (sucrose) decreased. Fructose was liberated in similar amounts in both samples. A discrepancy was noticed, namely that whereas the overall increase in spots 2–4 was 261 μg. fructose/20 μl., the disappearance of fructose from spot 1 (sucrose) was only 144 μg./20 μl. It did not seem possible that such a large difference could be caused by experimental error.

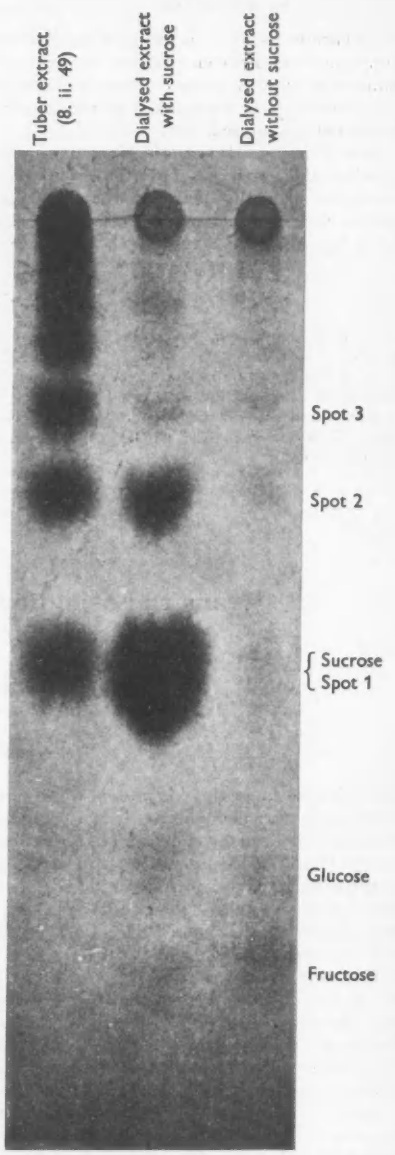

Fig. 1. Extract, prepared December 1949, dialysed 28 hr. against running tap water at 9°. Incubated 21 hr. at 26° with and without 10% added sucrose. Chromatogram run in butanol-acetic for 2½ days; sprayed with benzidine-trichloroacetic.

No glucose could be detected qualitatively in this experiment in spite of the sensitivity of the spraying reagent used (benzidine-trichloroacetic) which would have detected as little as 5 μg. glucose/20 μl. under the conditions used. A similar experiment to the one described, in which the presence of free glucose was investigated qualitatively and quantitatively, confirmed this result. Although the estimations of glucose were not very satisfactory (cf. Bacon & Edelman, 1951), it appeared from these and from the qualitative results that dismutation between sucrose molecules according to the equation

$$2(\text{glucose-fructose}) \rightarrow \text{glucose-(fructose)}_2 + \text{glucose}$$

could not account for the observations.

Table 1. *Incubation of tuber whole mash with added sucrose*

(Mash, prepared November 1949, incubated at 28° with 0·1 vol. 33% sucrose (w/v). No buffer added, pH about 6·5. Results of quantitative chromatographic analysis expressed as μg. ketose/20 μl. incubate, strained through gauze.)

	Time of incubation			
	0 hr.		27 hr.	
Spot	Sucrose added	Control	Sucrose added	Control
F	—	—	154	176
1	450	110	306	92
2	132	110	288	110
3	135	128	214	135
4	132	121	158	119
5 to *n*	1110	1050	800	920
Total	1959	1519	1920	1552

Elimination of extraneous causes. At this stage it was thought advisable to eliminate the possibility that the effects observed were due to contaminating bacteria, or to purely physical effects on the paper chromatogram.

(*a*) *Bacterial contamination.* In order to eliminate a heavy initial inoculum of micro-organisms tubers were scrubbed thoroughly with water and then with soap and water. They were peeled, washed with water, then with 70% ethanol, and the surface layers removed again. The remaining material was extracted in the usual way, chloroform being added at the start of disintegration in the Waring blender. Sucrose solutions saturated with chloroform were made up immediately before the tubers were extracted. Incubation was started a few minutes after the preparation of the extracts and stopped 2·5 or 5 hr. (Table 2) later. Chloroform was present throughout the incubations. A significant increase in spot 2 occurred in both cases. It is unlikely that bacteria caused the effect under these conditions during so short a period of incubation.

Earth chipped from the same batch of tubers was incubated at 37° with 7% sucrose in 0·004M-ammonium chloride. No material with R_F lower than sucrose was detected even after incubation for 52 hr. in the absence of chloroform. Traces of free glucose and fructose appeared under these conditions, but were not formed in the presence of chloroform.

Table 2. *Activity in absence of significant bacterial inoculum*

(See text for details. Extract, prepared January 1950, incubated at 30° with 0·125 vol. 40% sucrose (w/v) or water. No buffer added; pH about 6·5. Values expressed as μg. ketose/20 μl. incubate.)

	Time of incubation			
	0 hr.		5 hr.	
Spot	Sucrose added	Control	Sucrose added	Control
1	550	121	540	132
2	121	108	216	126
3	139	139	142	140
4 to *n*	1160	1160	1180	1130

All extracts used subsequently were made from peeled tubers washed thoroughly in water.

(*b*) *Physical interference on chromatograms.* The increase in spots 2–4 was not due to a physical effect of large amounts of sucrose on the chromatographic separation. Zero hour samples showed no significant differences from the controls (see Tables 2, 4, 8 and 9), boiled extract showed no increase of spot 2 in the presence of added sucrose (Table 3), and during the reaction there was a progressive increase of spot 2 with time of incubation (see below).

Table 3. *Effect of added sucrose on the chromatographic analysis of boiled extract*

(Sucrose added to 'standard substrate' (Edelman & Bacon, 1951).)

	μg. ketose/20 μl. solution	
Spot	Standard substrate with added sucrose	Standard substrate alone
1	485	150
2	150	146
3	160	160
4 to *n*	960	960

Effect of dialysis. Dialysis reduced the activity considerably, but did not destroy it entirely. This effect, first noticed qualitatively, is shown in Table 4, which shows also that dialysis for 53 hr. did not eliminate all the polysaccharide in the extract, the lower spots in the case of the dialysed extract incubated without sucrose probably being formed by the action of the hydrolytic system on the residual polysaccharide.

As compared with spot 2 glucose was formed in significant amounts in the presence of sucrose. However, not all dialysed extracts produced free glucose under these conditions.

Table 4. *Effect of dialysis on the reaction*

(Extract, prepared December 1949, dialysed 53 hr. at 9°. 1 vol. 50% sucrose incubated at 30° with 9 vol. of the dialysed liquid. No buffer added; pH about 6·7. Values expressed as μg. ketose/20 μl. incubate, except for 'spot *G*' which is expressed as μg. reducing substances (RS; calculated as hexose)/20 μl.)

	Time of incubation (hr.)											
	0		4		10		23		50		78	
Spot	Sucrose added	Control	Sucrose added	Control	Sucrose added	Control	Sucrose added	Control	Sucrose added	Control	Sucrose added	Control
F	46	56	57	62	77	79	91	112	139	130	176	156
G	0	0	0	0	0	0	35	0	44	0	58	0
1	500	11	505	11	525	20	432	35	424	23	490	26
2	225	245	28	9	49	12	66	14	57	23	44	13
3 to *n*			218	218	208	203	175	183	139	144	122	122
Total ketose	771	312	808	300	859	314	764	344	759	320	832	317

Table 5. *Restoration of activity to dialysed extract*

(See text for details. The pH of all incubations lay between 6·44 and 6·52. Values expressed as μg. ketose/20 μl. incubate.)

	Spot 2			Spot 3			
Incubation	Before	After	Increase	Before	After	Increase	Total Increase
1	73	146	73	75	81	6	79
2	74	145	71	75	79	4	75
3	64	129	65	62	68	6	71
4	66	126	60	62	70	8	68
5	123	203	80	110	132	22	102

Addition of boiled undialysed extract completely restored the activity. Two separate dialyses of the same extract were carried out for 9 and 22 hr. respectively against running water at 9°. Glass tubes containing undialysed extract were suspended in the running water for these periods. The dialysed extract increased in volume from 40 to 46 ml. (9 hr.) and from 40 to 49 ml. (22 hr.). The undialysed samples were diluted similarly. A sample (10 ml.) of each of the four extracts was placed in a boiling-water bath for 5 min. and then cooled. Five incubates were set up: (1) undialysed extract (2·5 ml.) + boiled dialysed extract (2·5 ml.) (9 hr. dialysis); (2) boiled undialysed extract (2·5 ml.) + dialysed extract (2·5 ml.) (9 hr. dialysis). Incubates 3 and 4 were set up, similarly to 1 and 2 but using the 22 hr. dialysed samples. Incubate 5 contained 2·5 ml. undialysed extract + 2·5 ml. boiled undialysed extract. To each incubate was added 1·0 ml. 30% sucrose solution to give an overall concentration of added sucrose of 5%. The results of analysis of the various components after 22 hr. incubation at 30°, given in Table 5, show that dialysed extract in the presence of boiled extract had the same activity as undialysed extract.

Attempts to concentrate the enzymic activity. An extract, prepared January 1950 (pH adjusted to 7 with N-sodium hydroxide), was fractionated between zero and 0·25, and 0·25 and 0·50 ammonium sulphate saturation by adding the solid (A.R.). The precipitates which formed were separated by centrifugation. Aqueous suspensions of these precipitates were dialysed overnight against running water and incubated at 28° with an overall concentration of 25% added sucrose at pH values of 6·8 (in 0·1M-phosphate buffer) and 5·0 (in 0·1M-acetate buffer). Samples taken at zero time showed no carbohydrate material on the chromatogram other than sucrose. In the case of the 0·25–0·50 saturation precipitate, later samples (5, 10, 28 and 53 hr.) showed traces of spot 2, glucose, and fructose, all becoming progressively more distinct in the course of time. These spots were weaker in the pH 5·0 incubate. The incubate containing the zero to 0·25 saturation precipitate showed only the slightest traces of these additional spots, and their appearance was delayed as compared with the 0·25–0·50 saturation precipitate. Even the greatest activity shown in this experiment, however, was negligibly small when compared with that of fresh tuber extracts. Further ammonium sulphate precipitates obtained between 0 and 0·60 saturation showed no detectable formation of spot 2 when incubated with sucrose, but their activity could be restored by adding boiled fresh extract. Such a precipitate, obtained from 300 ml. fresh extract, was taken up in water to give 15 ml., and this solution dialysed for 20 hr. against running cold water. To 4·0 ml. boiled fresh extract were added 0·8 ml. 30% sucrose solution and 1·0 ml. of this dialysed preparation, and to 4·0 ml. unboiled extract were added 0·8 ml. 30% sucrose and 1·0 ml.

Table 6. *Restoration of activity to ammonium sulphate concentrate by addition of boiled extract*

(For details see text. *A*, enzyme concentrate plus boiled extract. *B*, fresh extract. Concentration of added sucrose about 4%. No buffer added; pH about 6·7. Incubated at 30°. Values expressed as μg. ketose/20 μl. incubate.)

	Time of incubation					
	0 hr.		3 hr.		6 hr.	
Spot	*A*	*B*	*A*	*B*	*A*	*B*
F	29	32	47	42	61	47
1	553	553	517	517	480	493
2	88	95	166	142	211	176
3	92	88	103	102	108	109
4 to *n*	805	805	760	746	733	705
Total ketose	1567	1573	1593	1549	1593	1530

water. The mixtures were incubated at 30°. Results of analyses are given in Table 6; they showed that under these conditions the ammonium sulphate precipitate contained considerable activity. These experiments and those described above suggested that a thermostable co-factor was present in active preparations.

As the active system in tuber extracts could not be separated from the carbohydrates by dialysis or ammonium sulphate precipitation, further investigation was, of necessity, carried out on fresh extracts.

Further investigations of fresh extracts

Effect of varying pH *on the reaction.* The optimum lay between pH values 6·0 and 6·5 (see Table 7). As the pH of fresh extracts was in the neighbourhood of 6·5 no buffer was added in subsequent experiments.

Progress of the reaction with time. This is shown in Table 8. The following points were noticed: (1) Fructose is liberated in similar amounts both in the presence and absence of added sucrose. Although no figures were obtained for free glucose, this sugar was present in traces only (less than 5 μg./20 ml. incubate) and did not increase noticeably either in the presence of added sucrose nor during the progress of the incubation. (2) Ketose in spot 1 (sucrose) disappeared more rapidly in the incubate with added sucrose than in the control. This disappearance increased with increasing time of incubation.

Table 7. *Effect on* pH *on the increase in spot* 2

(Extract prepared 28 February 1950. pH adjusted with drops of N-HCl or N-NaOH. 1·0 ml. 30% sucrose was added to 5·0 ml. samples. Incubated at 30° for 4 hr. The amounts of spot 2 are represented by μg. ketose/20 μl.; the initial values at all other pH values are assumed to be equal to that for pH 7·0.)

	pH				
	7·0	6·5	6·0	5·5	5·0
Before	142	—	—	—	—
After	197	241	237	220	192
Increase	55	99	95	78	50

Table 8. *Progress of the reaction with time*

(Extract, prepared March 1950, incubated at 30° with 0·2 vol. 30% sucrose (w/v) or water. No buffer added. pH before incubation: of sucrose incubate 6·82, of control 6·84; after 20 hr. incubation: both 6·75. Values expressed as μg. ketose/20 μl. incubate.)

	Time of incubation (hr.)						Change in 18 hr.
Spot	0	1	2	4	7	18	
			(*a*) With added sucrose				
F	24	29	36	45	53	92	+ 68
1	666	654	642	636	606	570	− 96
2	150	178	201	243	278	357	+207
3	165	176	168	182	176	194	+ 29
4 to *n*	1214	1204	1158	1158	1114	1068	−146
Total ketose	2219	2244	2205	2264	2227	2271	—
			(*b*) Control				
F	23	30	37	46	57	102	+ 79
1	156	153	150	150	148	148	− 8
2	133	144	147	165	156	166	+ 33
3	155	159	155	167	169	184	+ 29
4 to *n*	1220	1204	1220	1200	1296	1120	−100
Total ketose	1687	1690	1769	1728	1826	1720	—

(3) The ketose content of spot 2 increased throughout the time of incubation, very much more in the incubate with added sucrose than in the control. (4) There was no difference between the rates of increase of the ketose contents of spot 3 in the two incubates. (5) There was some indication that the ketose content of spots 4 to *n* was decreasing more rapidly in the presence of added sucrose than in the control. (The fluctuation of the ketose content of this group of spots was much larger in absolute terms than that of the lower spots because much larger total amounts were being estimated; measurement of the decrease, which was of the same order as the change in spot 1, was therefore subject to a much larger experimental error.) (6) The amount of combined fructose which disappeared from spot 1 approximated fairly closely to half the fructose appearing in spots 2 and 3 together.

From (5) and (6) above, the possibility was suggested that the material causing the increase in spots 2 and 3 was derived partly from spot 1 and partly from higher spots.

Effect of varying sucrose concentration. The effect of varying concentrations of sucrose on the reaction was therefore investigated in an attempt to establish a more reliable quantitative relationship between the changes in ketose content of the various components of the system. The results of such an experiment are given in Table 9, which shows good agreement between the total ketose estimated before and after incubation, the greatest discrepancy being about 3% and the average 1%. Fructose was liberated in similar amounts at all levels of added sucrose, there being a slight decrease with increasing concentration of added sucrose at the higher levels. The increase in ketose content of spot 3 was also similar at all levels of added sucrose concentration except the two highest. These variations in spots *F* and 3 are sufficiently small to have little effect on the calculations of the changes in spots 1, 2 and 4 to *n*, in making which it has been assumed that the ketose appearing in spots *F* and 3 was derived ultimately from spots 4 to *n*. In the table, therefore, all material higher than spot 2 has been treated as a single entity, namely spots 3 to *n*.

When the amount of spot *F* is added to the values for spots 3 to *n* there remains still a significant amount of ketose to be accounted for (Table 9*b*, iii), an amount increasing with increasing sucrose concentration, and approximately equal to the corresponding decrease in spot 1 (Table 9*b*, iv, v). It should be noted that small percentage errors in analysis would cause very large errors in the ratios given in (v), particularly at the extremes of sucrose concentration, since with no added sucrose the error in measuring spots 3 to *n* is large in comparison with

Table 9. *Effect of varying sucrose concentration on the reaction*

(Extract prepared March 1950. Incubated at 28° with 0·2 vol. sucrose solution of appropriate concentration. No buffer added; the pH values of all incubates lay between 6·53 and 6·57. All values expressed as μg. ketose/20 μl. incubate.)

		Concentration of added sucrose as percentage (w/v)													
		Zero		0·5		1·0		1·5		3·0		5·0		8·5	
(*a*) Analyses:															
Spot *F*		33*	79†	35	75	35	81	36	79	36	77	35	74	33	69
1		155	144	203	178	248	213	299	252	427	345	595	485	957	910
2		137	163	137	185	141	212	143	236	145	278	152	312	149	355
3		138	155	135	162	140	162	144	171	138	164	146	162	155	160
(4 to *n*)		1095	1007	1095	997	1083	986	1075	957	1099	957	1095	925	1077	947
Total		1558	1548	1605	1597	1647	1654	1696	1695	1845	1821	2023	1958	2371	2441
(*b*) Calculations:															
Increase in spot *F*	(i)		46		40		46		44		41		39		36
Decrease in spots (3 to *n*)	(ii)		70		71		75		91		116		154		125
Decrease in spots (3 to *n*) corrected for material appearing in spot *F*, i.e. (ii) − (i)	(iii)		24		31		29		47		75		115		89
Decrease in spot 1	(iv)		6		25		35		47		82		110		47
Ratio (iii)/(iv)	(v)		4·00		1·24		0·83		1·00		0·92		1·05		0·53
Sum of decreases in spot 1 and spots (3 to *n*) i.e. (iii) + (iv)	(vi)		30		56		64		94		157		225		136
Increase in spot 2	(vii)		26		48		71		93		133		160		206
Ratio (vi)/(vii) (average of these values 1·07)	(viii)		1·15		1·17		0·90		1·01		1·18		1·41		0·66

The left-hand* and right-hand† columns for each concentration of added sucrose refer to values before and after incubation, respectively.

the increase in spot 2, and with high concentrations of sucrose the absolute errors in measuring the ketose contents of both spots 1 and 3 to n become considerable. Thus, an error of 5 % in the estimation of spot 1 in the samples containing no added sucrose could cause an error in this ratio of about 55 %, whilst a similar error in estimation of the sample containing the highest level of added sucrose could cause an error of about 100 %.

If the results with these two samples are ignored, the average value of the ratio $\frac{\text{decrease in spots 3 to } n}{\text{decrease in spot 1}}$ is 1·01.

These results confirmed the previous assumption that the material appearing as spot 2 was derived both from sucrose and from the polysaccharide material contained in spots 4 to n (Table 9*b*, vi, vii and viii). A hypothesis in accordance with the facts presented is that fructose residues are transferred from polysaccharide to sucrose molecules, forming a trisaccharide in the first instance:

$$(\text{fructose})_n + \text{glucose-fructose} \rightarrow (\text{fructose})_{n-1} + \text{glucose-(fructose)}_2.$$

Further fructose residues might then be transferred to the trisaccharide, yielding a tetrasaccharide, and so on.

Action on mixtures of inulin and sucrose

Preparation of active concentrates. According to the above hypothesis, systems not containing a fructose donor (i.e. polysaccharide) would not show the reaction, which would explain why no significant amounts of oligosaccharide had been produced when ammonium sulphate precipitates were incubated with sucrose alone. The traces sometimes formed under such conditions may have been due to the presence of a small amount of undialysable polysaccharide from the original extract.

Confirmatory evidence in support of the hypothesis was obtained by an experiment in which a mixture of sucrose and inulin was incubated with an ammonium sulphate fraction (prepared between 0·25 and 0·55 saturation). Spot 2 was formed; when the enzyme preparation was incubated with inulin alone, or sucrose alone, it was not (see Fig. 2).

The preparation used in this experiment had been concentrated with respect to its hydrolytic activity (see Edelman & Bacon, 1951), and its transferring activity was fairly weak. It was found that addition of ammonium sulphate to 0·60 saturation in fresh extracts precipitated material which, when taken up in a small volume of water and dialysed, showed rather higher activity as judged qualitatively in systems containing inulin and sucrose. In later experiments spots 2 and 3 and traces of higher spots were produced in such systems. Although the reaction was usually carried out in the presence of about 0·02 M-phosphate buffer (pH 6·5) inorganic phosphate did not appear to be necessary for the production of the higher spots. Similar systems set up in the presence and absence of phosphate buffer showed no difference in activity as judged qualitatively.

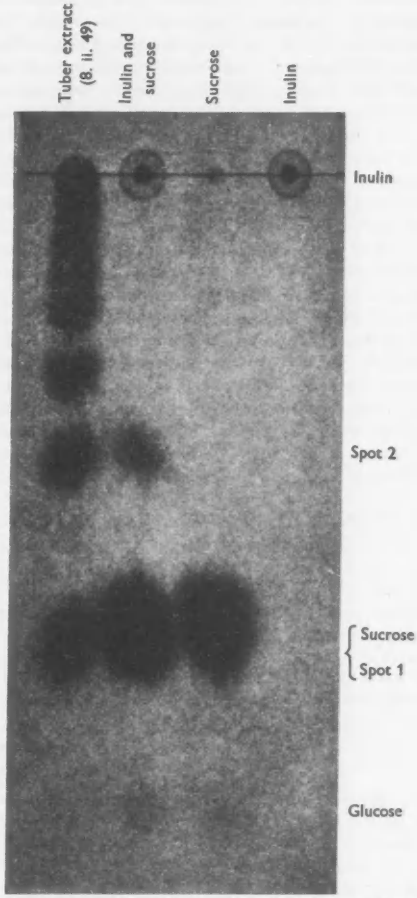

Fig. 2. Enzyme preparation, made February 1949, incubated with 2·5 % added inulin, with 5 % added sucrose, and with both, for 24 hr. at 30° in 0·1 M-phosphate buffer (pH 6·5). Chromatogram run in butanol-acetic 2 days; sprayed with benzidine-trichloroacetic.

The same reaction was found to occur with a 0·60 saturation with ammonium sulphate precipitate of an aqueous extract of Dahlia tubers.

Monosaccharide liberation. The enzyme preparations just described were from extracts made towards the end of the storage season (end of May–June) and were found to liberate free glucose and fructose from

sucrose. Such dialysed preparations on centrifugation at high speed (i.e. about 10,000 *g*) gave a copious precipitate of material which had not redissolved during dialysis. The clear supernatant liquid contained the transferring activity but very little sucrose-hydrolysing activity, most of which was contained in the precipitate. For example: an enzyme preparation, prepared June 1950, was dialysed for 18 hr. against running cold water. Part of the dialysate (7·0 ml.) was centrifuged at high speed for 10 min. The clear yellow supernatant liquid was decanted (5·3 ml.), the precipitate washed twice with water by centrifugation and the slightly opalescent washings added to the original supernatant liquid. This solution was made up to 10·6 ml. with water. The precipitate was suspended in water and made up to 7·0 ml. These two preparations were incubated in a solution containing 2·5 % inulin and 5 % sucrose, and in a solution of 5 % sucrose alone. Similar incubations were carried out using the original dialysed preparation. The incubates contained 0·02 M-phosphate buffer (pH 6·5). The results are given in Table 10, and indicated that the transferring system and part of the monosaccharide liberating system were independent of each other. Unless otherwise stated, all the preparations having transferring activity referred to below had been subjected to high-speed centrifugation.

Table 10. *Distribution of transferring and fructose-liberating activities*

(Dialysed concentrate centrifuged at about 10,000 *g* for 10 min. Supernatant liquid combined with washings, and washed precipitate, tested. Incubated in 0·02 M-phosphate buffer (pH 6·5) for 18 hr. at 30°. Values expressed as mg. produced by the equivalent of 10 ml. original concentrate. Substrate *A*: 5 % sucrose (w/v); substrate *B*: 5 % sucrose (w/v/) plus 2·5 % inulin (w/v).)

Enzyme	Liberation of free fructose: Substrate *A*	Liberation of free fructose: Substrate *B*	Ketose content of spots formed with $R_F <$ sucrose $>$ inulin from substrate
Original whole concentrate	108	177	221
Supernatant liquid and washings	29	39	227
Precipitate	105	131	52·5

Effect of varying concentrations of sucrose and inulin. Variations in the concentrations of sucrose and inulin had a considerable effect upon the transferring activity at the levels tested (enzyme preparation not centrifuged); see Table 11. The free fructose liberated by the hydrolytic system was not affected by variation in sucrose concentration.

Degree of concentration of enzymic activity. In Table 6 the increase in spot 2 during 6 hr. incubation with 4 ml. fresh extract was 81 μg. ketose/20 μl. incubate; after 6 hr. incubation with 1 ml. enzyme concentrate it was 123 μg. The degree of concentration of activity was thus (4 × 123):81 or about 6:1. A calculation based on the figure for fresh extract with 5% sucrose in Table 9 and that for the concentrate with 2% inulin and 5% sucrose in Table 11 shows no appreciable concentration, but in this case the comparison may not be valid, since there was four times as much fructose in carbohydrates other than sucrose in the former incubation.

Table 11. *Effect of varying concentrations of inulin and sucrose on the activity of an enzyme concentrate*

(Ammonium sulphate concentrate (overall dilution × 5); incubated 18 hr. at 30° in about 0·01 M-phosphate buffer (pH 6·5) with various concentrations of inulin and sucrose. Values expressed as μg. ketose/20 μl. incubate.)

Inulin (%)	Sucrose (%): 1·0	3·0	5·0
	(*a*) Formation of spot 2		
0·5	13	20	24
2·0	19	40	55
	(*b*) Formation of spot *F*		
0·5	55	57	49
2·0	143	157	151

Isolation of spot 2 formed from inulin and sucrose. An enzyme preparation (10 ml.) was incubated at 30° in a total volume of 30 ml., containing 1·5 g. sucrose, 1·5 g. inulin, and 0·03 M-phosphate buffer (pH 6·5). After 20 hr. a slight turbidity was seen, presumably due to the precipitation of some of the inulin. After 24 hr. 2 vol. of ethanol were added, and the mixture filtered after 30 min. The precipitate was washed with 65% ethanol and the filtrate and washings evaporated to dryness under reduced pressure with a water-bath temperature of 40°, yielding 1·75 g. of yellow syrup. 1·58 g. of this were placed on a column of 75 g. Whatman Ashless Tablet cellulose powder, 35 cm. high and 2·8 cm. in diameter, and the chromatogram developed with *n*-butanol-N-aqueous ammonia mixture (Hough, Jones & Wadman, 1949). Examination of the effluent by paper partition chromatography showed that sucrose was present between 1180 and 3015 ml. of total effluent, the last 755 ml. of this fraction containing also spot 2. The fraction between 3015 and 4600 ml. contained only spot 2 and after 4600 ml. both spots 2 and 3.

Part of the effluent containing only spot 2 (855 ml.) was taken to dryness as before, the syrup taken up in 50 % ethanol and treated with charcoal. When again taken to dryness it consisted of 165 mg. of slightly yellow syrup, which could not be induced to crystallize. It was analysed without fractionation (see Table 12). The remainder of the spot 2 fraction (in aqueous butanol) was reduced to a volume of

about 20 ml., when the precipitate that appeared was separated by centrifugation, washed with *n*-butanol and absolute ethanol, dried *in vacuo*, and analysed (Table 12); this material would appear to have been relatively free from the inert material which contaminates the effluent. The ratio of fructose:glucose of about 2 : 1 calculated from these analyses supports the view that spot 2 is a trisaccharide formed by the addition of a fructose residue to sucrose.

Table 12. *Analyses of spot 2 formed from inulin and sucrose*

(All weights are expressed as mg.)

		Unfractionated material from column	Residue after extraction with *n*-butanol
Dry weight of syrup	(i)	137	14·6
RS before acid hydrolysis	(ii)	8·4	0·32
RS after acid hydrolysis*	(iii)	92·5	13·4
Total ketose	(iv)	58·5	8·7
Total RS as percentage dry weight from (i) and (iii)		67·5	92
Free RS as percentage total RS from (ii) and (iii)		9·1	2·4
Ratio fructose : glucose from (iii) and (iv)		1·7 : 1	1·9 : 1
Ratio fructose : glucose from (iii) – (ii) and (iv)		2·3 : 1	2·0 : 1

* Corrected from fructose standard, assuming one-third glucose.

Specificity of the enzymic action

The replacement of inulin in the system by ethanol fractions of the tuber carbohydrates. The possibility that various ethanol fractions of the tuber carbohydrates might give a greater effect than inulin was first investigated. Fractions precipitated between 0–80, 70–80 and 80–90 % ethanol, were prepared by adding the appropriate volume of ethanol to fresh extract, prepared April 1950, and allowing the solutions to stand for 2 hr. at room temperature. The supernatant liquid was decanted and the sticky precipitates (a syrup in the case of the 80–90 % fraction) well drained; the 0–80 % fraction was reprecipitated twice from 80 % aqueous ethanol. The fractions were taken up in water. Both the 70–80 and 80–90 % fractions contained considerable amounts of carbohydrate with R_F greater than zero, there being very little material with zero R_F in the latter case. The 0–80 % fraction contained no material with R_F appreciably greater than zero. Qualitative chromatographic comparison of incubates after 15 hr. at 30° in 0·02 M-phosphate buffer (pH 6·5), containing these fractions (at 5 % concentration of combined ketose) together with sucrose (5 %) and an enzyme preparation (1 vol. in 3; prepared May 1950), showed a somewhat greater increase of spot 2 in the case of the 70–80 and 80–90 % fractions as compared with a similar system using inulin. The 0–80 % fraction, however, showed a rather lower activity than inulin. There being no very striking differences between these systems and those containing inulin, it was not considered profitable to use ethanol fractions routinely as fructose donors in place of inulin, because the 70–80 and 80–90 % fractions were considerably contaminated with spots 2 and 3, etc., and because inulin is a more definitely characterized material than any obtained by precipitation between arbitrarily defined limits of ethanol concentration (cf. Dean, 1904).

Replacement of sucrose by hexose. Analogous reactions involving glucose or fructose would be expected to give, in the first instance, disaccharide.

An enzyme preparation, prepared June 1950, showed no formation of intermediate carbohydrate material when incubated with a mixture of glucose and inulin. With a mixture of fructose and inulin, however, spots were formed, each of which, although very similar in position to spots 1, 2 or 3 on butanol-acetic acid chromatograms, had a somewhat greater R_F than the corresponding tuber component (see Fig. 3). Similar results were obtained when glucose was present in addition to fructose and inulin. Control incubations of enzyme with glucose and/or fructose in the absence of inulin, and inulin in the absence of hexose, showed no such results.

The compounds formed by the reaction between inulin and fructose resembled spots 1, 2, etc., in being attacked by invertase, but differed in containing no combined glucose. Thus their reaction with benzidine-trichloroacetic was typical of fructose in giving a weak yellow instead of the strong chocolate-brown colour typical of glucose; the spots showed strongly with the phloroglucinol reagent. Conclusive evidence for the absence of combined glucose was obtained by two-dimensional chromatography, invertase being used to hydrolyse the spots after the first development; this hydrolysis gave rise to fructose alone.

When treated with an acetone solution of silver nitrate, and then with ethanolic sodium hydroxide as described by Trevelyan, Procter & Harrison (1950) the spots showed a much slower reducing action than fructose; sucrose and raffinose spots on the same chromatogram reacted at the same time although less intensely. This could not be taken as evidence that they were non-reducing, since turanose also reacted almost as slowly as sucrose.

The di- and trisaccharide appearing during the early action of hydrolytic enzyme preparations on inulin (Edelman & Bacon, 1951) behaved similarly with respect to all these tests and are thus probably due to a reaction between the free fructose formed from inulin, and unchanged inulin.

Replacement of sucrose by other oligosaccharides. Using 1 vol. of an uncentrifuged enzyme concentrate in 3 with inulin (5%) as fructose donor, lactose, maltose and trehalose (all 5%) showed chromatographically no differences after incubation (up to 30 hr.) from control incubations with these sugars in the absence of inulin. The incubations were carried out in 0·02M-phosphate buffer (pH 6·5). There was some lactase and maltase activity and a considerable trehalase activity as judged qualitatively.

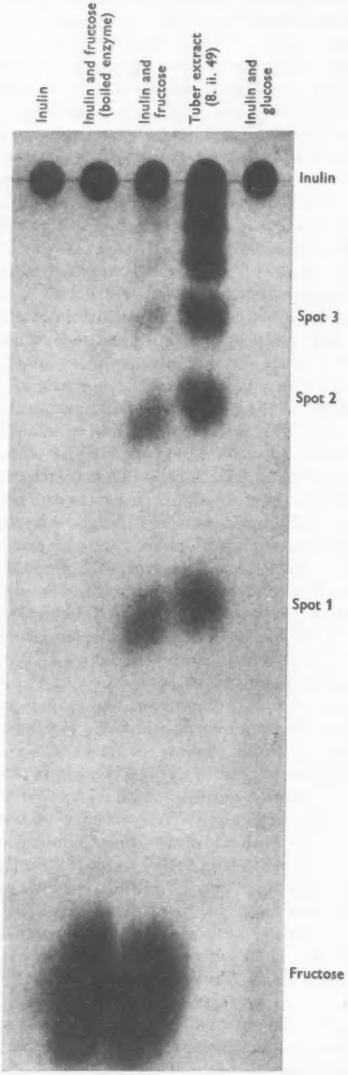

Fig. 3. Enzyme preparation, made June 1950, incubated with various combinations of 5% added inulin, 7·5% added fructose and 7·5% added glucose for 41 hr. at 30° in 0·1M-phosphate buffer (pH 6·5). Chromatogram run in butanol-acetic for 2 days; sprayed with phloroglucinol.

However, in a similar experiment using raffinose (7·5%), there appeared after 5 hr. a spot with R_F smaller than that of raffinose, and with approximately the same intensity as spot 2 formed during a parallel incubation with sucrose of about the same molar concentration (0·15M, i.e. 5%, w/v). On continued incubation (30 hr.) a further spot, with still smaller R_F, appeared. (Enzyme preparations after centrifugation still contained some melibiase activity, although the invertase activity had been eliminated.)

Experiments with melezitose (kindly supplied by Dr C. S. Hudson of Bethesda, Maryland, U.S.A.) also gave positive results. Incubates containing enzyme preparation (1 vol. in 3), 5% inulin and 5% melezitose in 0·02M-phosphate buffer (pH 6·5) showed, after 18 hr. at 30°, a spot with R_F smaller than that of melezitose on chromatograms run in butanol-acetic or phenol. Incubation in the absence of inulin showed no such spot; the melezitose was not hydrolysed.

Replacement of inulin by other polysaccharides. Various polysaccharides were incubated with the enzyme (uncentrifuged; 1 vol. in 3) and sucrose (5%). The incubations were carried out in 0·02M-phosphate buffer (pH 6·5).

Spots appeared with R_F smaller than that of sucrose with both starch and glycogen, but these spots also appeared in the controls incubated without sucrose. In the case of glycogen the spot immediately above sucrose (presumably maltose) was stronger in the control, and there was a series of weaker spots extending as a streak to the starting line both in the presence and absence of sucrose. These spots (reducing dextrins?) all gave the reddish colour typical of maltose with the benzidine-trichloroacetic spraying reagent. Small amounts of glucose were liberated from both starch and glycogen.

A grass levan (kindly supplied by Dr D. J. Bell) showed no spots other than those of the carbohydrates added, either in the presence or absence of sucrose. Irisin 'B' (dialysed before use) showed a trace of a spot in position '2' after 30 hr. incubation in the presence of sucrose. The spot did not appear in the absence of sucrose.

DISCUSSION

The experiments described show the presence in extracts of artichoke tubers of an enzyme system which catalyses the transference of fructose residues from inulin and associated polysaccharides to sucrose. No evidence, however, is available to show

how small the polysaccharide donor or how large the oligosaccharide acceptor may be; it is not excluded that relatively small molecules may take either part in the reaction, although sucrose does not donate fructose to sucrose to any appreciable extent.

The latter observation calls attention to the similarities in specificity (as far as this has been tested) between the hydrolytic enzyme system described previously (Edelman & Bacon, 1951) and the donor side of the transfructosidation system. The specificity of the acceptor side of the transfer reaction is low, requiring only a fructose residue, free or combined. (One acceptor, melezitose, is not attacked hydrolytically by the enzyme preparation studied.)

The question therefore arises whether the same enzyme is responsible for both the hydrolytic and the group-transfer reactions. Such a view might receive support from recent observations on other enzymes attacking hydrolysable substrates. For example, Rabaté (1935, 1937) described transglycosidation in plant extracts which were capable of hydrolysing certain heterosides; Axelrod (1948*a*, *b*) and Appleyard (1948) described transphosphorylation catalysed by acid phosphatase preparations; and Fruton (1950; Johnston, Mycek & Fruton, 1950) described a transpeptidation reaction with cysteine-activated papain. Fruton (1950) suggested a mechanism to account for his observations, and remarked 'it will be important to examine the possibility that some of the enzymes considered to function solely as catalysts in hydrolytic reactions may have an important physiological role in the catalysis of replacement reactions' (Johnston *et al.* 1950). This view was further borne out by the observation of Meyerhof & Green (1950) that alkaline phosphatase would catalyse transphosphorylation reactions, and those of Blanchard & Albon (1950) and Bacon & Edelman (1950) that yeast invertase preparations formed substances presumed to be trisaccharides during their hydrolytic action on sucrose.

If one assumes that such dual activities are the property of a single enzyme it may be postulated that the primary action of the enzyme is to combine with the substrate, which may be represented as *A*-*B*. Three (or perhaps more) possibilities then present themselves: (1) that the elements of water may combine with the substrate, so that hydrolysis takes place, (2) that *B* (and less probably *A* as well) may be exchanged for a substance of similar structure (*C*) yielding the compound *A*-*C*, (3) that *B* may be exchanged with a phosphate radical, the substance *A*-phosphate being formed.

In the case of acid phosphatases only the alcohol or phenol moiety is exchangeable, and water may take its place. With sucrose phosphorylase only the fructose residue is exchangeable, either by other sugars (L-sorbose, D-xyloketose, L-arabinose), or by phosphate, but not by water (cf. Hassid & Doudoroff, 1950). All three reactions may be regarded equally as group-transfer reactions: to water, to an organic acceptor, or to phosphate, respectively.

Wholly hydrolytic enzymes and wholly transferring enzymes would on this assumption represent merely the extremes of a general type. In the case of certain enzymes that attack sucrose one may discern a gradation extending from yeast invertase, which shows relatively slight transferring activity, through the mould invertases, which show appreciable transfer (Edelman, Bacon & Bealing, 1950), to levansucrase (Hestrin *et al.* 1943; Hestrin & Avineri-Shapiro, 1944) which produces a polysaccharide and only very little free fructose.

Changes in pH might alter the ratio of the hydrolytic to group-transfer activities even if these were the property of a single enzyme. Thus Johnston *et al.* (1950) found that at a higher pH transpeptidation was increased relative to hydrolysis. Axelrod (1948*a*), however, found little difference in the pH optima for the two activities of his orange juice phosphatase preparation. In artichoke tuber extract the pH optimum of transfructosidation is higher than that for hydrolysis of the tuber carbohydrates.

A consequence of (1) and (2) is that there should be competition between water and the other acceptors in the system. Since the concentration of water is already at its maximum in an aqueous medium, increase in the absolute concentration of another acceptor would mean an increase in its concentration relative to that of water. This is consistent with the increase in transfructosidation with increasing sucrose concentration shown in Table 9, and might explain the high molar concentrations apparently needed for maximum transfer, which has not so far been achieved in our experiments.

Whether the rate of hydrolysis should alter in the presence of acceptors other than water cannot be predicted. It is conceivable that hydrolysis might be reduced by the presence of an acceptor (cf. orange juice phosphatase/methanol/methyl phosphate system, Axelrod, 1948*a*; also Rabaté, 1937) or increased if the product of group-transfer were more readily hydrolysed (cf. prostatic acid phosphatase/*iso*propanol/phenolphthaleinphosphate system, Appleyard, 1948). The latter possibility recalls the earlier observations of Behrens & Bergmann (1939) on the 'co-substrate' effect of certain peptides with proteolytic enzymes. The fact that the liberation of free fructose does not alter much with a fivefold increase in acceptor concentration and a tenfold increase in the rate of transfructosidation thus cannot be taken as evidence that the two systems are independent.

From these considerations it follows that the question of the identity of the hydrolytic and

fructose-transferring enzyme system of the artichoke tuber remains an open one. However, the existence of a transfructosidation reaction lends further support to the view that the structures of all the components of the tuber carbohydrates are based upon that of sucrose (Bacon & Edelman, 1951; cf. also Bell, 1947).

SUMMARY

1. From artichoke tubers enzyme preparations have been obtained which catalyse the transference of fructose residues from inulin and associated polysaccharides to sucrose, thereby forming a trisaccharide and higher oligosaccharides.

2. Evidence has also been obtained for the transference of fructose residues to raffinose, melezitose, and to free fructose, but not to maltose, lactose, trehalose or glucose.

3. The liberation of free hexose which took place in all the preparations examined does not appear to be connected quantitatively with the group-transfer reaction.

4. The reaction, for which the name 'transfructosidation' is proposed, is discussed in the light of existing knowledge of hydrolytic and group-transferring enzyme systems.

Note added 22 *June* 1951. Since this paper was submitted our attention has been drawn to the work of Dedonder (1950, 1951), from which he drew independently conclusions similar to our own about the nature of the artichoke fructosans, and suggested that phosphate was necessary for their synthesis from sucrose.

This work was done with the technical assistance of Mr R. Loxley. We wish to thank Dr K. Burton and Prof. H. A. Krebs, F.R.S., for discussions of the wider significance of our results; and Dr C. S. Hudson for gifts of melezitose and turanose. One of us (J.E.) is indebted to the Agricultural Research Council for grants during the course of the research.

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The Construction of a Counter-current Apparatus

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The introduction of an apparatus for counter-current extraction by Craig (1944) was a notable contribution to the separation of a wide variety of compounds. Recently Craig (1950) has devised a glass apparatus of large capacity.

In applying the counter-current principle to the separation of lipids it has been found necessary to design a new apparatus in which the fluid phases could easily be examined for emulsions and removed for centrifuging, if necessary. It was desirable that the instrument be suitable for both large and small volumes of fluid. These characteristics have been embodied in an apparatus of glass and plastic with 49 'theoretical plates'. Its simplicity, and low cost, would seem to adapt it to general laboratory use.

General description of apparatus

The apparatus (Figs. 1, 2) consists of an array of 50 pairs of vertical wide glass tubes with conical ends supported by 'Terry' clips on either side of a wooden frame. The lower ends of each pair are joined by narrow polythene tubing, and neighbouring pairs are joined at the upper ends by wide polythene tubing, the whole forming a continuous spiral around

the frame. Every second glass tube (i.e. all the glass tubes along one side of the frame) has a short open side arm. A bolt at each end of the frame permits the array to be rotated about a horizontal axis.

Each pair of tubes (Fig. 2) corresponds to a 'theoretical plate'. The tubes without side arms (Fig. 2, *A*) are referred to as mixing tubes, and those with a side arm (Fig. 2, *B*) as transfer tubes. To operate the apparatus the two phases are equilibrated in the mixing tube by rocking the central frame about the horizontal position. Separation into two phases is carried out in the mixing tube, and when the tubes are placed in the vertical position the heavy phase flows into the transfer tube. Rotation of the transfer tube over the mixing tube moves the heavy layer on to the next mixing tube. This cycle is repeated, once for each of 50 pairs of tubes, which are numbered 0 to 49.

Details of construction

The general plan of the apparatus is given in Fig. 1, and details of one pair of tubes in Fig. 2. The pairs of tubes are spaced at intervals of 3·5 cm. between centres. The dimensions of the apparatus are adapted to the use of phases of 5–40 ml., of either equal or unequal volume. With the larger volumes the first pair of tubes cannot be used, as tube 0*A* has a side arm.

Several aspects of the construction merit particular mention. Glass tubing must be selected so that it makes a good fit with the polythene tubing available commercially. For the wide plastic tube (Fig. 1, *D*) we use 22 cm. of polythene tubing of 6 mm. bore (Telegraph Construction and Maintenance Co. Ltd.). Our glassblowers select the wide glass tubing (external diameter 6·5 mm.) so that it makes a snug

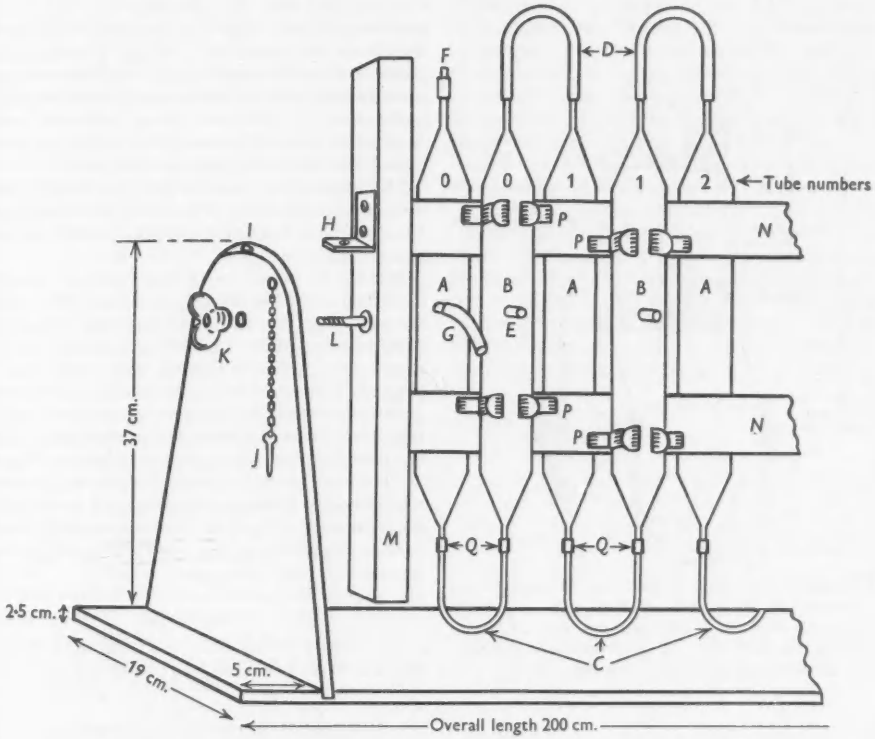

Fig. 1. Schematic diagram of 49 'plate' counter-current apparatus. The tubes are here spread out to show the spiral connexions. *A*, mixing tubes; *B*, transfer tubes; *C*, narrow polythene tubing; *D*, wide polythene tubing; *E*, short open side arm; *F*, short polythene tube and glass rod; *G*, mixing tube with side arm; *H*, metal angle on handle of central frame; *I*, hole; *J*, dropping pin; *K*, butterfly nut; *L*, bolt permitting rotation; *M*, handle of central frame; *N*, wooden frame; *P*, Terry clips; *Q*, joints between tubes and polythene connexions (for details, see Fig. 2).

fit with the wide polythene tubing. The penalty for too tight a fit is cracking of the polythene.

The narrow plastic tubing (Fig. 1, *C*), 23 cm. long, is polythene tubing (internal diameter 1·5 mm., external diameter 2·5 mm.; Polythene Cannula, size 3, from Allen and Hanbury Ltd.). This tubing has been selected because its volume is small, but it is not so narrow that flow is greatly retarded. The dimensions of the narrow tubing preclude attachment over glass tubing. In order that the glass can be sufficiently large to be strong, the narrow polythene tubing is attached to glass of 2·5 mm. internal and 3·5 mm. external diameters, the joint being made by two polythene sleeves. The outer (Fig. 2, *F*) has an internal diameter of 3·5 mm. (No. 55, Polythene Tubing from Portland Plastic Ltd.). The inner (Fig. 2, *G*) has an external diameter of 3·7 mm. and an internal diameter of 2·5 mm. (Telegraph Construction and Maintenance Co. Ltd.). The narrow glass tube (Fig. 2, *H*) may be drawn off the wide tube, or sealed on, though this appears to produce a weaker attachment. Should this latter procedure be used, it is important to select glass by trial with the polythene tubing available, rather than to rely on measurements alone. The polythene-glass joints are sturdy and solvent proof.

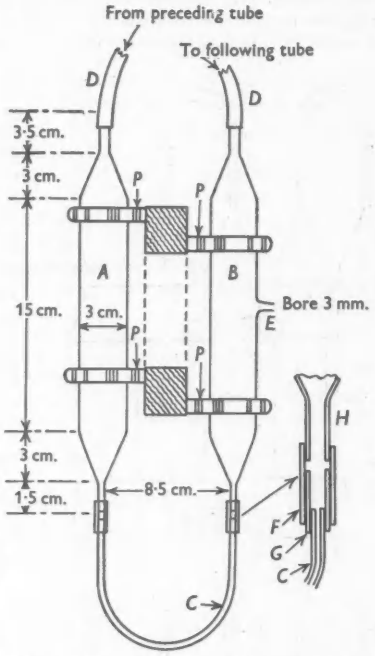

Fig. 2. Sectional view of one pair of tubes. *A*, mixing tube; *B*, transfer tube; *C*, narrow polythene tubing; *D*, wide polythene tubing; *E*, short open side arm; *F*, outer polythene sleeve of joint; *G*, inner polythene sleeve of joint; *H*, narrow glass tube; *P*, Terry clips.

The taper of the ends of the glass tubes is satisfactory when made according to the drawing (Fig. 2). More acute taper leads to trapping of fluid, when the tubes are turned.

While tube pairs 1 to 48 are of uniform construction, 0 and 49 differ slightly because they represent the ends of the spiral. In the first pair *A* is not connected to any other tube by its wide end, which is closed by a short polythene tube and glass rod (Fig. 1, *F*). To allow for rapid introduction of solvent, *A* is constructed similarly to *B*, with a side arm (Fig. 1, *G*) which is extended by polythene, a distance of about 8 cm., to the right-hand surface of tube *B*.

The end of the spiral, tube *B* of pair 49, is not connected by its wide end. This ends in a stopcock of 3 mm. bore, the barrel of which is secured by a small rubber ring and peg. The heavy layer of pair 49 is removed through this tap.

Manipulation

To begin a distribution with the apparatus, the required amounts of the two phases are equilibrated and separated. The manipulation of the apparatus will be described as if the observer were standing at the end of the apparatus, with tube *B* on the right, and *A* at the left.

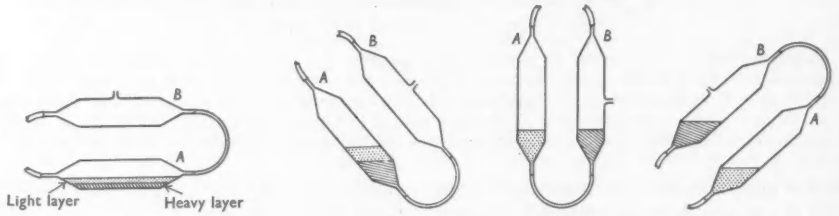

Fig. 3. Diagrams showing positions of tubes during manipulation. *a*, '9 o'clock' (tubes are shaken); *b*, '10 o'clock' (allowed to settle); *c*, '12 o'clock' (height of *B* adjusted); *d*, '8 o'clock' (transfer to next tube).

The apparatus is rotated so that the wide plastic tube is at the 8 o'clock position (Fig. 3*d*). At this point a metal angle (Fig. 1, *H*) on the handle of the central frame prevents further rotation by impinging on the upright. The two phases, containing the substance to be studied, are run into tube *A* of pair 0 via the side arm of *A* (Fig. 1, *G*). We find it convenient to deliver the phases from a 20 ml. glass syringe, to which is attached a short length of narrow polythene tubing, which passes through the side arm.

To equilibrate the two phases, the central wooden shaft is rotated back and forth about 25° on either side of the horizontal plane (Fig. 3*a*) so that the two phases flow from one end of the mixing tube *A* to the other. In practice it is found best to dip the narrow end of the tubes rather more than the wide, since otherwise liquid may be trapped in the wide tube. This rocking procedure is carried out for 30–60 sec., at a rate of about 70/min. After mixing, the central frame is clamped by the butterfly nut (Fig. 1, *K*) with the wide plastic tubing in a 10 o'clock position (Fig. 3*b*).

When the phases have separated the central shaft is turned to the 12 o'clock position (Fig. 3*c*), and secured there by dropping a pin (Fig. 1, *J*) through *H* into a hole *I*. The heavy layer then moves into transfer tube *B* through the narrow polythene tubing. When this movement is almost complete, transfer tube *B* is raised a little in the Terry clips (Fig. 1, *P*) so that movement stops with the meniscus in the plastic tubing, close to the lowest point, and on side *A*. Once this level has been set, it should not require further adjustment unless the relative volumes of the two phases alter considerably.

In order to move the heavy layer on by one tube, the central shaft is rotated counter-clockwise, to the 8 o'clock position (Fig. 3*d*). This rotation is made smoothly but quickly, in order that the heavy layer should not pass back through the narrow plastic tube into mixing tube *A*. The cycle is completed in 3–4 min.

Further cycles are now made in a similar way, but with this difference that the new light phase is run into the mixing tube with the advancing heavy layer. This is done via the side arm of the previous transfer tube, since in the present position (Fig. 3*d*) this leads down through the wide plastic tube to the next mixing tube. Following mixing and separation, when the heavy layer moves from *A* to *B*, only the levels of the two leading transfer tubes have to be adjusted.

A distribution of 49 plates requires approximately 3 hr. In the usual standard procedure the apparatus is turned into the 12 o'clock position and the contents of each pair of tubes are now removed by suction, through narrow polythene tubing introduced via the side arm.

To carry out procedure 1 of Craig (1944), once the tubes are full the procedure is continued, with the variation that in each cycle one new heavy layer is introduced into mixing tube 0, and one removed from transfer tube 49. This is continued until a desired constituent begins to appear in the heavy layer removed.

In procedure 2 of Craig, the number of cycles is increased until most of the desired material has passed out with the heavy layer. With the apparatus described above, it is possible to run as many plates as is desired. For instance, to run 99 plates on an apparatus with 50 pairs of tubes the standard procedure is followed as above until the apparatus is full. The process is continued, as in Craig's procedure 1, until 50 additional heavy layers have passed through. As these layers come out they are numbered (50–99) and kept separate. The apparatus is then emptied and the first 50 fractions are ready for analysis. The second 50 plates are now run, as in the standard procedure, using new upper layers and introducing the heavy layers into mixing tube 0 in the order which they came out of the apparatus. It will of course be appreciated that bottom layer 50 will become 99 at the conclusion of the distribution and vice versa. The run is complete when tube 49 (now 99) has been reached again.

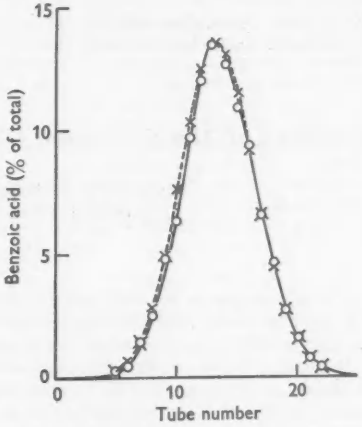

Fig. 4. Comparison of experimentally determined distribution (○——○) of benzoic acid ($K = 2{\cdot}22$) in a run of 44 'plates', with theoretical distribution (×– – –×) for $K = 2{\cdot}22$.

One troublesome feature with some solutes is a tendency to emulsification, or in the case of some lipids, even gel formation. Should it be necessary to remove the phases from one tube for spinning, this is readily done in the vertical position by sucking out both phases through the side arm. The apparatus may even be run with three or four tubes removed for

shaking by hand and centrifuging. In this case the appropriate layers have to be withdrawn and returned to the apparatus at each stage.

The apparatus may be used for two 24 plate distributions at the same time, one beginning at tube 0 and one at tube 26.

Effect of solvents on plastic. Although polythene plastics are not entirely resistant to organic solvents, their limitations for our purposes seem few. Mixtures containing light petroleum, benzene, ethyl ether, chloroform, carbon tetrachloride, methylene chloride, methanol and other alcohols have been used.

Faint longitudinal streaks in the narrow plastic tubing and cracks at the joints are occasionally seen, but the tubing is readily replaced.

Performance

As a test of the performance of the apparatus we have employed benzoic acid, in a similar way to Craig (1944). As solvent we have used a mixture of 62 ml. of carbon tetrachloride, 35 ml. of methanol and 3·15 ml. of water. After equilibration the heavier phase is approximately 41% (v/v) of the whole. The distribution constant (K) of benzoic acid in this mixture has been found by titration to be 2·22, at a concentration of 0·5% (w/v), the two phases being of equal volume. Using phases of 20 ml., and 0·196 g. of benzoic acid, a distribution of 44 plates was run, and the amount of benzoic acid in each pair of tubes determined by titration with 0·1 N-sodium hydroxide, using phenolphthalein as an indicator. For comparison, the theoretical distribution of a solute of $K = 2·22$ has been calculated by formulae (3) and (4) from Williamson & Craig (1947). The experimental and theoretical distributions, which are presented in Fig. 4, are in close agreement.

SUMMARY

1. A 49 plate counter-current apparatus of simple construction in glass and plastic is described.

2. Using benzoic acid as a test substance, the performance of the apparatus approximates closely to a theoretical distribution.

Our thanks are due to Mr E. C. Clark, for preparation of line drawings, to Aimer Products Ltd., for care in selection of glass and for the glassblowing, and to Mr R. Spratley, for skilful carpentry. Dr J. Murray, under whose direction the work has been done, has given us every encouragement during the development of the apparatus.

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Turacin and the Supposed Catalytic Activity of Copper-Porphyrins

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The role of free copper in the catalytic oxidation of ascorbic acid in foods was first demonstrated by Hess & Ungar (1921–2) and was later confirmed by Euler, Myrbäck & Larsson (1933). The mechanism of this oxidation was investigated by Barron, Meio & Klemperer (1936), who found that ascorbic acid is not autoxidizable in acid and neutral solutions, but that under these conditions it is rapidly oxidized by molecular oxygen in the presence of copper. This reaction is specific for copper and may be used for detecting concentrations of the metal as low as 46 μg./l. The catalytic oxidation of cysteine in pyrophosphate buffer was used by Warburg (1927) for detecting and estimating copper in blood serum, and this method was found to be both extremely sensitive and specific for copper.

In the course of their investigations on the oxidation of ascorbic acid, Stotz, Harrer & King (1937) found that the reaction took place in the presence of copper-haematoporphyrin and that it was inhibited by sodium diethyldithiocarbamate. Haemin (iron-protoporphyrin) failed to catalyse the oxidation of ascorbic acid. Salomon (1939) claimed that copper-protoporphyrin could catalyse the oxidation of cysteine, its activity on a copper basis being 18·3% of that of copper sulphate at the optimum pH (7·8). On the basis of these results and of those of Stotz *et al.* (1937), it was suggested by Salomon that catalysis by copper-porphyrin might occur without change in valency of the copper.

The main object of this paper is the re-investigation of this problem using turacin, which is the only known naturally occurring copper-porphyrin. Turacin, which constitutes the red pigment of the wing feathers of certain species of the African Turaco bird, was first described by Church (1869, 1892). It was

shown to be a copper-uroporphyrin by Fischer & Hilger (1924), who believed that the uroporphyrin belongs to series I, but Rimington (1939) proved that it is, in fact, copper-uroporphyrin III.

Turacin is freely soluble in dilute alkali giving a purple-red solution with a two-banded absorption spectrum in the visible region. The positions of the absorption bands are shifted towards the red end of the spectrum on acidification, and this acid type of spectrum is given by the feather itself. It was shown by Keilin, D. (1926) that this variation in the position of the absorption bands depends on the degree of dispersion of turacin, since acidification causes molecular aggregation of the pigment, which, in the absence of a protective colloid, proceeds to precipitation. This effect may be reversed by the addition of solvents such as ethanol or acetone; caffeine also disperses the pigment (Keilin, J., 1943), and in all these cases the absorption bands change to the alkaline type even in acid solution. The absorption spectra in the visible and near violet regions were determined to see how the alteration in the state of dispersion of the pigment affects the γ-band (Soret band), and also to establish a standard by which to determine the concentration of the turacin solutions used in the investigations described in this paper.

In view of the inability of turacin to react with many substances which combine with iron-porphyrin compounds (Keilin, D., 1926) and the drastic chemical measures needed to remove the copper from the porphyrin ring (Fischer & Hilger, 1924) it was decided to see whether turacin could be chemically altered in any way by bacteria (*Bacillus subtilis*) or by moulds (*Penicillium notatum* and *Aspergillus niger*); the fate of the pigment was also studied after parenteral administration in the rat.

EXPERIMENTAL

Turacin. The red wing feathers of a Turaco bird were well washed in several changes of ether to remove the grease. After drying, the pigment was extracted by two or three washings in 0·2 N-NH_4OH, and the solution was filtered and acidified with a few drops of glacial acetic acid. The bulky brown precipitate was centrifuged off and washed twice with distilled water; the turacin was then redissolved in 0·2 N-NH_4OH. For the manometric experiments the acid-precipitated turacin was washed repeatedly with twice glass-distilled water and finally dissolved in the appropriate Cu-free buffer. It was found that two feathers yielded 21·8 ml. of a dark-red $3{\cdot}73 \times 10^{-4}$ M-turacin solution (based on the Cu content of the solution determined by the method of Eden & Green, 1940). This solution was diluted 1 in 40 with 0·2 N-NH_4OH, and the absorption spectrum in the visible and near violet regions was measured. This absorption curve was used as a standard for determining the concentrations of other turacin solutions.

Haematoporphyrin. This was taken from a stock preparation and dissolved in 0·01 N-NaOH to give a solution containing 4 mg./ml. (9×10^{-4} M).

Spectroscopic observations. For all qualitative spectroscopic examinations a microspectroscope was used. More detailed study of absorption spectra was carried out with a Beckman photoelectric spectrophotometer using 1·0, 0·5 and 0·25 cm. glass cells. The molecular extinction coefficient is defined as $\epsilon = E/cl$, where c = molarity of the turacin solution, l = optical depth and E (extinction) = $\log I_o/I$, where I_o and I are the intensities of the incident and transmitted light respectively.

Culture media. Peptone broth was used for *B. subtilis*, and the Czapek-Dox medium was used for *P. notatum*. *A. niger* was grown on a medium containing 1% (w/v) $(NH_4)_2SO_4$, 0·1% (w/v) K_2HPO_4, 0·05% (w/v) $MgSO_4$ and 4·0% (w/v) sucrose in distilled water. For each organism a series of six Erlenmeyer flasks was set up each containing 50 ml. medium. A solution of turacin was added to flasks 2–6 of each series so that the final concentrations of the pigment in these flasks ranged from 0·0052 to 0·026 mg. turacin/ml. medium. Flask 1 of each series served as control. The flasks were autoclaved, inoculated with the appropriate organism and incubated at 37°.

Manometric experiments. Barcroft differential manometers were used at 39°. The total amount of fluid in each manometer flask was 3·3 ml. After equilibration in the usual way the ascorbic acid or cysteine was introduced by dislodging the dangling cups and the O_2 uptake was measured. The Q_{O_2} (μl. O_2/mg. Cu/hr.) was calculated on the basis of the initial velocity of the reaction. To eliminate as far as possible the catalytic action of extraneous Cu and Fe, all glassware was cleaned in conc. HNO_3 followed by tap water (known to have a low metal content) and then with twice glass-distilled water until the rinsings gave a dark-green colour when tested with a solution of dithizone in CCl_4. Twice glass-distilled water was used for preparing all solutions. Ascorbic acid (Roche Products) was weighed directly into the dangling cups. A very white preparation of cysteine hydrochloride was found to give satisfactory results without any further purification. It was dissolved in Cu-free pyrophosphate buffer, pH 7·6, the solution was adjusted to this pH with NaOH and diluted with the buffer to a final concentration of 33·4 mg. cysteine/ml. solution. Pyrophosphate buffers were prepared from twice recrystallized sodium pyrophosphate and redistilled HCl.

RESULTS

Absorption spectrum of turacin

The positions of the absorption bands in the visible region of the spectrum of acid and alkaline turacin (Table 1, Figs. 1 and 2) are in close agreement with those previously given (Keilin, D., 1926). Of the two additional bands at 597 and 484 mμ. described by MacMunn in Church's (1892) paper, only the second band was seen.

In order to determine at what pH the absorption spectrum of turacin changes from the alkaline to the acid form, the absorption densities of turacin solutions containing 0·4% (w/v) gelatin and ranging from pH 10 to 2·87, were determined spectrophotometrically at 563, 560 and 520 mμ. respectively, and the percentage of turacin in the alkaline form in each solution was calculated using the formula of Roche & Morena (1936). The results show (Fig. 3) that turacin

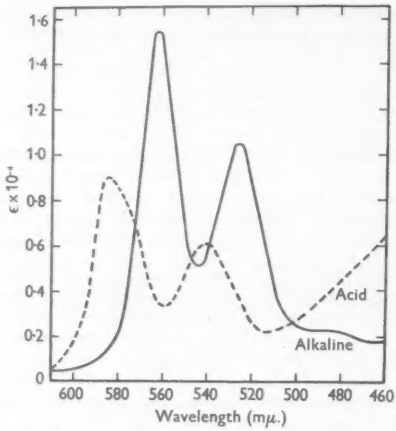

Fig. 1. Absorption bands in the visible region of the spectrum of alkaline turacin and acid turacin in 0·4% (w/v) gelatin. Turacin = $5{\cdot}69 \times 10^{-5}$ M. $l = 0{\cdot}5$ cm. (ϵ as defined in text).

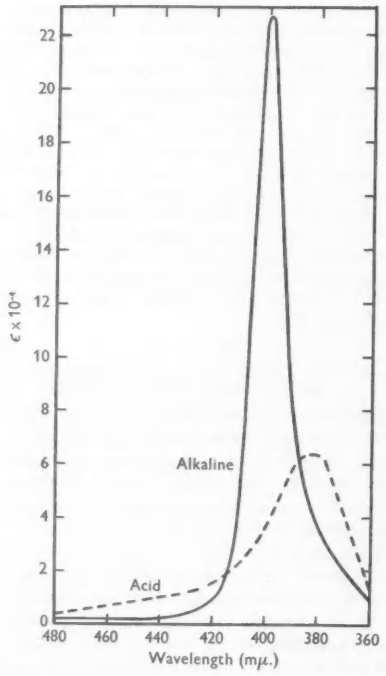

Fig. 2. Absorption spectra showing the γ- (Soret) bands of acid and alkaline turacin. Turacin = $5{\cdot}69 \times 10^{-5}$ M for acid turacin in 0·4% (w/v) gelatin and $1{\cdot}138 \times 10^{-5}$ M for alkaline turacin. $l = 0{\cdot}5$ cm.

has a pK of 5·65 and that below pH 4·7 only the acid type of spectrum is seen.

Action of solubilizing agents on the Soret band of porphyrin compounds

The variation of the absorption spectrum of a pigment with its degree of dispersion was first described by Stenger (1888). Svedberg (1909*a*, *b*) and Ostwald (1911, 1918) also studied this problem and found that the greater the degree of dispersion of a pigment the nearer its absorption bands lie to the violet end of the spectrum.

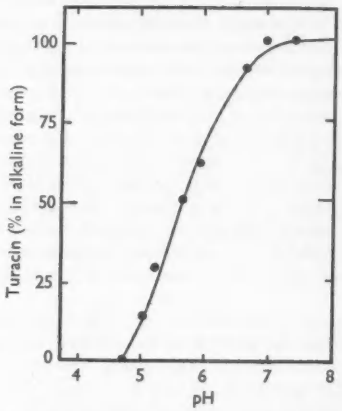

Fig. 3. Percentage of turacin present in the alkaline form as a function of pH.

The spectra of alkaline and acid turacin show that the α- and β-absorption bands follow this rule, as was demonstrated by Keilin, D. (1926), but that in acid turacin, where there is marked molecular aggregation, the γ-band (Soret band) lies 23 mμ. nearer the ultraviolet end of the spectrum than does the γ-band of alkaline turacin. On the addition of caffeine to acid turacin, the α- and β-bands are shifted towards the violet end of the spectrum, and this is also the case on addition of solvents such as ethanol or acetone, whereas the γ-band moves in the opposite direction (Table 1). There is, however, a slight anomaly in the action of caffeine on alkaline turacin. Here the α- and β-bands are very slightly shifted towards the red end of the spectrum, but this change in position of 1·5 mμ. may be due mainly to increased salt concentration. The shift of the γ-band in the opposite direction to the movement of the α- and β-bands was also observed on increasing the solubility of alkaline mesoporphyrin, protohaem and carbon monoxide protohaem (Table 2).

These results show that the generally established relationship between the positions of the absorption

Table 1. *Positions (to nearest 0·5 mμ.) and heights of the absorption bands in the visible and near-violet regions of the spectrum of acid and alkaline turacin under different conditions*

(Alkaline turacin: $5{\cdot}69 \times 10^{-5}$ M in 0·2 N-NH_4OH. Acid turacin in gelatin: $5{\cdot}69 \times 10^{-5}$ M (0·5 ml. of $2{\cdot}84 \times 10^{-3}$ M-turacin and 5 ml. glacial acetic acid were diluted to 25 ml. with 0·2 N-NH_4OH containing 0·2% (w/v) gelatin to prevent the precipitation of the pigment). Acid turacin + caffeine: the gelatin was omitted and the solution contained instead 500 mg. caffeine per 25 ml. solution. Alkaline turacin + caffeine: $5{\cdot}69 \times 10^{-5}$ M-turacin in 0·2 N-NH_4OH containing 500 mg. caffeine per 25 ml. solution.)

	α-Band		β-Band		γ-Band	
	λ (mμ.)	$\epsilon \times 10^{-4}$	λ (mμ.)	$\epsilon \times 10^{-4}$	λ (mμ.)	$\epsilon \times 10^{-4}$
Alkaline turacin	562	1·54	526	1·04	399	22·7
Acid turacin in gelatin	583·5	0·90	540	0·61	382	6·4
Acid turacin + caffeine	564	1·48	527·5	0·92	405	21·3
Alkaline turacin + caffeine	563·5	1·73	527·5	0·94	405·5	23·2

Table 2. *The effect of solubilizing agents on the positions of the 'Soret' bands of turacin, mesoporphyrin, protohaem and* CO*-protohaem*

Compound	Solubilizing agent	Position of Soret band (mμ.)	Shift towards red (mμ.)
*Acid turacin (in gelatin)	None	382	—
Alkaline turacin	None	399	17
Acid turacin	Caffeine	405	23
Alkaline turacin	Caffeine	405	6
Mesoporphyrin (in 0·1% Na_2CO_3)	None	373	—
	Caffeine	399	26
Protohaem (in 1% Na_2CO_3)	None	389	—
	Ethanol	416	27
CO-protohaem (in 1% Na_2CO_3)	None	406·5	—
	Ethanol	410	3·5

* Turacin solutions as in Table 1.

bands and the state of dispersion of the pigment applies only to the α- and β-bands and not to the Soret band which moves in the opposite direction to the bands in the visible region of the spectrum.

The effect of living systems on turacin

(*a*) *Bacteria and moulds. B. subtilis* grew well and the turacin remained in solution since the pH of the medium was maintained around 7·3. After about 2 weeks the organism was filtered off using a Seitz filter, and the turacin was recovered from the medium by acid precipitation followed by solution in dilute alkali. Spectroscopically the pigment was unchanged. The organism was subcultured on agar slopes and continued to grow normally in every way.

Penicillium notatum and *Aspergillus niger*. Both cultures passed normally through all stages of their development. The medium became sufficiently acid to precipitate the turacin, but on isolation and redissolution in alkali it was found, on spectroscopic examination, to be unchanged.

(*b*) *Rats.* Two half-grown female litter mates were kept in metabolism cages fitted with a device for separating the urine from the faeces. The animals were fed on rat cake and given free access to water. 1 ml. of a neutralized solution containing 2·4 mg. turacin was injected subcutaneously in the abdominal wall of one rat. Within 80 min. of the injection the urine became pink and 11·2 ml. of bright-pink urine were collected in the first 21 hr. after injection. This urine and that of the control rat were cleared by filtration followed by high-speed centrifugation. The absorption spectrum of the excreted alkaline turacin was identical with the standard spectrum, and the concentration of turacin in the pink urine was measured using the control urine as 'blank'. About 2·2 mg. turacin were accounted for, and since the rat continued to excrete slightly pink urine for several hours more, it is possible that the total quantity injected was excreted. Injections of turacin, about 2 mg. per dose, were given every third day until a total of 7 mg. had been given; the animal remained in good condition and continued to gain weight until it was sacrificed 4 weeks later.

There is, therefore, good evidence that turacin cannot be broken down by any of the organisms examined. If indeed any copper had been liberated from the turacin it would have had marked toxic effects on the moulds which are extremely sensitive to free copper.

It has been shown by Schultz, Elvehjem & Hart (1934) that anaemic rats requiring copper for haemopoiesis are unable to make use of copper fed to them as copper-haematoporphyrin which was passed into the faeces unchanged so far as could be determined spectroscopically. These authors also found that copper-haematoporphyrin was non-toxic to rats, since animals receiving available copper as well as copper-haematoporphyrin in their food recovered normally.

Copper-uroporphyrin and the oxidation of ascorbic acid and cysteine

The oxidation of ascorbic acid in the presence of copper sulphate as catalyst was studied first to establish the optimum conditions for the reactions before examining the effect of turacin. Pyrophosphate buffer was used as described by Salomon (1939), but the rate of oxidation was found to be extremely slow. It was therefore decided to use orthophosphate buffer instead, and in the presence of 0·1 M-orthophosphate, pH 6·5, the Q_{O_2} of copper

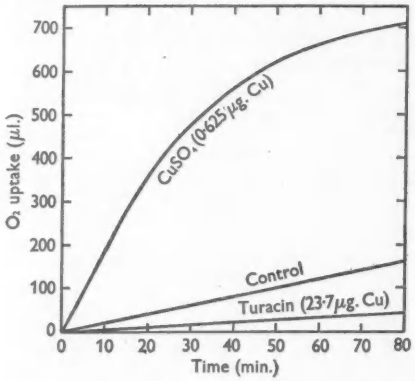

Fig. 4. Oxidation of ascorbic acid ($1{\cdot}44 \times 10^{-2}$ M) in 0·1 M-orthophosphate buffer, pH 6·5, in the presence of $CuSO_4$ ($2{\cdot}98 \times 10^{-5}$ M), turacin ($1{\cdot}13 \times 10^{-4}$ M) and haematoporphyrin ($9{\cdot}0 \times 10^{-4}$ M). The values obtained with haematoporphyrin are the same (within experimental error) as those obtained in the presence of turacin. Total volume 3·3 ml.; $t = 39°$.

reached a value of 1,560,000. The experiment was then repeated using turacin instead of copper sulphate, and it was found that turacin (containing 23·7 μg. copper as copper-porphyrin) was completely devoid of any catalytic activity in this system and even depressed the rate of autoxidation of ascorbic acid (Fig. 4).

The effect of a free porphyrin (haematoporphyrin) was examined as well as that of turacin since Salomon (1939) admitted the presence of free porphyrin in his copper-porphyrin preparation which had a four-banded absorption spectrum. The same results were obtained with haematoporphyrin as with turacin.

The oxidation of cysteine in the presence of copper sulphate was carried out in 0·2 M-pyrophosphate buffer, pH 7·6 and the Q_{O_2} of the copper-catalysed reaction was found to be 1,416,000. Turacin, however, had a negligible effect on the oxidation of cysteine (Fig. 5).

DISCUSSION

Copper is universally distributed in the plant and animal kingdoms and forms an integral part of many substances which show great diversity in their biological properties and functions. Thus it forms the active group of haemocyanin, the respiratory pigment of many molluscs and arthropods, and also of haemocuprein and hepatocuprein which are found in mammalian blood and liver respectively, though

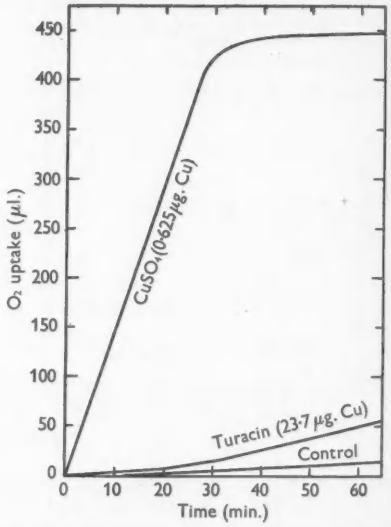

Fig. 5. Oxidation of cysteine ($2{\cdot}5 \times 10^{-2}$ M) in 0·26 M-pyrophosphate buffer, pH 7·5, in the presence of $CuSO_4$ ($2{\cdot}98 \times 10^{-5}$ M) and turacin ($1{\cdot}13 \times 10^{-4}$ M). Total volume 3·3 ml.; $t = 39°$.

their functions are as yet unknown (Mann & Keilin, D., 1939). Copper is also the active group of the enzymes laccase, tyrosinase and ascorbic acid oxidase. In all these compounds the metal is bound directly, though relatively loosely, to the protein, and it has been shown that the copper may be removed from tyrosinase and laccase by treatment with cyanide followed by dialysis to remove the copper-cyanide complex (Kubowitz, 1938; Tissières, 1948).

Turacin is the only known example of a naturally occurring copper-porphyrin and it is of extremely limited distribution. It may be noted here that whereas turacin is only found free, its iron counterpart, iron-porphyrin (haem) is always found bound to protein (Keilin, D. & Hartree, 1947).

Additional evidence is given here to show that the most characteristic property of turacin is its stability and complete inability to react with other substances. Thus it is unaffected by reducing and

oxidizing agents (Keilin, D., 1926), or by growing organisms such as *B. subtilis* and the moulds *P. notatum* and *A. niger* to which the pigment is not toxic. So far as could be determined by spectroscopic and spectrophotometric measurements turacin was unchanged during its absorption into the blood-stream and excretion by the kidneys in the rat, but further work would still be needed to check the integrity of all eight carboxyl groups of the porphyrin ring.

Change of valency in a metalloporphyrin is accompanied by changes in the visible absorption spectrum. The absorption spectra of haem (Fe^{++}) and haematin (Fe^{+++}) are well known and marked differences in the absorption spectra of the bi- and ter-valent manganese and cobalt mesoporphyrin compounds have been demonstrated by Taylor (1940). No alteration in the absorption spectrum attributable to changes in valency have been observed in turacin under any conditions. Moreover, it was found that turacin is unable to catalyse the oxidations of either cysteine or ascorbic acid.

These findings cannot be reconciled with the claims made for the catalytic properties of copper-haematoporphyrin by Stotz *et al.* (1937) and for copper-protoporphyrin by Salomon (1939), and indeed they supply additional evidence for believing that a valency change in the copper is essential if it is to act as an oxidizing catalyst.

In their studies on the characterization of cytochrome oxidase, Eichel, Wainio, Person & Cooperstein (1950) state that there is direct evidence that cytochrome oxidase is a copper-containing enzyme. This view is based on the following facts: reduced cytochrome oxidase (in the presence of sodium deoxycholate) has two well defined absorption bands at 601 and 440 mμ. and Eichel *et al.* (1950) found that there is good correlation between the height of the 601 mμ. band and the copper content, but not with either the iron content or the activity of the preparations. Since the heights of the bands at 440 and 601 mμ. in reduced cytochrome oxidase bear a constant ratio to each other, the obvious inference is that the 440 mμ. absorption band is also proportional to the copper content of the preparation. This band, however, is the Soret band which is characteristic of a porphyrin ring, and one would therefore have to postulate that cytochrome oxidase is a copper-porphyrin-protein compound. Since, as is shown in this paper, the only naturally occurring copper-porphyrin (turacin) is catalytically inactive and its copper does not combine with protein, it is extremely unlikely that a copper-porphyrin can be a prosthetic group of an enzyme or respiratory carrier analogous in structure to the haemoproteins. It remains to be seen whether the copper compound found by Eichel *et al.* to accompany cytochrome oxidase has any catalytic activity in the cytochrome oxidase system. The spectrum given by these authors of cytochrome oxidase is that of cytochrome a_3, which is undoubtedly an iron-porphyrin-protein compound (Keilin, D. & Hartree, 1939).

SUMMARY

1. Turacin (copper-uroporphyrin III) was found to be unable to catalyse the oxidation of either ascorbic acid or cysteine.

2. In view of these findings, the great stability and unreactivity of turacin, and the fact that its copper does not combine with protein, it is difficult to visualize how a copper-porphyrin could act as a prosthetic group of an oxidizing catalyst.

3. The catalytic activity of copper-porphyrins obtained by other workers can probably be attributed to traces of free copper left over from the preparation of these compounds.

4. Spectrophotometric studies were made of acid and alkaline turacin in the visible and near-violet regions of the spectrum. It was shown that on increasing the degree of dispersion of the pigment, as for example with caffeine, the Soret band is shifted towards the longer wavelengths, i.e. in the opposite direction to the well known shift of the bands in the visible region of the spectrum. These findings were found to apply to several other porphyrin compounds examined.

5. Turacin was not affected by bacteria (*Bacillus subtilis*), moulds (*Penicillium notatum* and *Aspergillus niger*) or by passage through the kidney in the rat after parenteral administration.

I wish to thank Dr E. Hindle, F.R.S., and Dr R. E. Rewell for Turaco feathers from the Zoological Gardens, London, Dr C. Mann for her co-operation in carrying out the rat injections and Mr B. H. Baker for the cultures of bacteria and moulds. A part of this work was done while receiving a personal grant from the Medical Research Council.

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Effects of High-voltage Cathode Rays on Aqueous Solutions of Histidine Monohydrochloride

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It has been reported by Proctor & Bhatia (1950) that irradiation of haddock fillets by cathode rays caused no significant loss of amino-acids in the protein. The investigation reported was limited to ten amino-acids, namely, arginine, histidine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine and cystine. The observations indicated that these amino-acids either were not affected in the concentrations in which they were present in the fish protein, or that they were protected from the action of cathode rays (electrons) when present in native environments.

Although there are reports in the literature relating to the irradiation of histidine by ultraviolet rays and by X-rays, no reference to the effect of cathode rays is known. Ellinger (1928) reported that irradiation of histidine hydrochloride with ultraviolet radiation led to the formation of a pharmacologically active product with histamine-like action. Further irradiation at 2970 and 3020 A. resulted in a loss in pharmacological activity. This work was confirmed by Holtz (1933, 1934), who isolated crystalline histamine picrate. Investigations conducted by Szendrö (1931) indicated that imidazole acetaldehyde is also formed from histidine. Splitting off of ammonia from a number of amino-acids by light of the quartz mercury lamp has been measured by Lieben & Molnar (1931) and by Lieben & Urban (1931, 1934). It was indicated that the rate of decomposition of histidine by irradiation is independent of pH. Dale, Davies & Gilbert (1949) reported that 10·6 μg. NH_3/ml. were liberated from 0·13 M solution of histidine monohydrochloride after exposure to an X-ray dose of 166,000 r.

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The present report describes experiments conducted to determine the chemical and physical effects of cathode rays on histidine, and presents evidence regarding the mode of action of cathode rays on histidine.

EXPERIMENTAL

Source of cathode rays. A pressure-insulated, electrostatic generator of the Van de Graaff type, operating at 3,000,000 V. and capable of producing a continuous supply of monoenergetic electrons, was used for the production of the cathode rays (Robinson, 1947; Trump & Cloud, 1943; Trump & Van de Graaff, 1948). The dosages of cathode rays are expressed in terms of röntgen-equivalent-physical (r.e.p.) (Evans, 1947).

Irradiation. Samples (10 ml. each) of the amino-acid solution were irradiated in stainless-steel dishes 5·08 cm. in diameter and 0·8 cm. in depth. The depth of the liquid when the sample amounted to 10 ml. was 0·5 cm. This is well within the penetration range of electrons at the voltages used. The dishes were uncovered during irradiation and all irradiations were made at room temperature. After irradiation, the samples were transferred to glass bottles.

Estimation of histidine. Determinations of histidine were made microbiologically, *Leuconostoc mesenteroides* P-60 being used as the test organism. The basal medium 'D' developed by Dunn, Shankman, Camien, Frankl & Rockland (1944) was used.

For chemical analysis of histidine, a colorimetric method (Macpherson, 1942; Block & Bolling, 1945) based on a modification of Pauly's (1904, 1915) reaction was used. The red colour was measured in an Evelyn colorimeter having a 515 mμ. filter. A standard curve of histidine monohydrochloride monohydrates, covering a range of 0–100 μg., was prepared.

Estimation of deamination. For the observations on deamination, 3 ml. samples of histidine solution, prepared in 0·1 N-HCl, were sealed into 5 ml. glass ampoules, which were subjected to the cathode-ray beam. After irradiation, the

tip of the ampoule was broken and the liquid was withdrawn for NH_3 analysis. NH_3 was determined by the micro-diffusion method (acidimetric) as described by Conway (1947). The test fluid (1 ml.) was put in the central chamber of the standard unit. NH_3 was absorbed in 0·001 N-HCl. Non-irradiated histidine solution was taken as the control in the blank unit.

Ultraviolet absorption. The ultraviolet absorption spectra of the irradiated and the non-irradiated amino-acid solutions were determined with a Beckman quartz spectrophotometer model DU.

RESULTS

Disappearance of histidine on irradiation. A yellow pigmentation was produced in histidine solutions upon irradiation. The intensity of the colour increased progressively with dosage at a given concentration and also with concentration at a given dosage.

Four aqueous solutions of histidine containing 100, 250, 500 and 1000 μg. of the amino-acid per ml. of solution were irradiated. The results on the amounts of unchanged amino-acid as determined microbiologically are shown in Table 1.

Two irradiations (I and II) were conducted on two different days, and the amounts of unchanged amino-acid in each sample were measured both chemically and microbiologically. The results are shown in Table 2. Comparisons of the data presented in Tables 1 and 2 indicate the degree of variation that may be encountered in this type of irradiation and the subsequent evaluation of the results.

Production of ammonia. The yields of ammonia obtained on irradiation of solutions of histidine monohydrochloride containing 100 μg./ml. at four levels of 100,000, 250,000, 500,000 and 1,000,000 r.e.p. are presented in Table 3.

Ultraviolet absorption spectra. Fig. 1 shows the ultraviolet absorption spectra of histidine monohydrochloride solutions (100 μg./ml.) irradiated at different dosages. The increased absorption by the irradiated solutions indicates that the molecule of histidine is affected by irradiation.

DISCUSSION

Inspection of Table 1 shows that histidine monohydrochloride was decomposed upon irradiation with cathode rays. The difference in the amino-acid content before and after irradiation has been considered as representing the amount decomposed on irradiation. The decomposition of amino-acid over

Table 1. *Effect of high-voltage cathode rays on aqueous solutions of* L-*histidine hydrochloride hydrate*

Dosage (r.e.p.)	Percentage retention of amino-acid in solutions with concentrations per μg. per ml. of: 100 (pH 4·71)	250 (pH 4·48)	500 (pH 4·34)	1000 (pH 4·18)
Control (non-irradiated)	100·0	100·0	100·0	100·0
100,000	44·5	52·4	66·8	80·7?
250,000	17·5	35·9	61·3	62·7?
500,000	6·0	29·2	53·2	64·3
1,000,000	0·0	12·4	28·2	43·1

Table 2. *Effect of high-voltage cathode rays on aqueous solutions of* L-*histidine monohydrochloride hydrate* (100 μg./ml.)

Dosage (r.e.p.)	Percentage retention of histidine as determined: Chemically Run I	Run II	Average	Microbiologically Run I	Run II	Average
100,000	58·8	60·3	59·6	48·4	51·3	49·9
250,000	38·9	31·5	35·2	21·1	20·2	20·7
500,000	20·0	12·3	16·2	8·0	7·2	7·6
1,000,000	7·0	1·3	4·2	0·0	0·0	0·0

Table 3. *Deamination of histidine monohydrochloride* (100 μg./ml. *in* 0·1 N-HCl) *by irradiation with high-voltage cathode rays*

Dosage (r.e.p.)	Ammonia N (μg./ml.) Replicates	Average	NH_3 (μg./ml.)
100,000	3·00, 2·82, 3·60, 3·48	3·22	3·90
250,000	5·31, 5·25, 5·70, 5·70	5·49	6·66
500,000	7·35, 7·32, 7·38	7·35	8·92
1,000,000	9·36, 9·46, 9·06, 9·12	9·25	11·23

the range of concentrations studied was related exponentially to the dosage. This was found to be a first-order reaction. With an equal dosage of irradiation, the dilute solution was relatively more affected than the more concentrated solutions. This observation demonstrates the 'dilution phenomenon' in radiochemical reactions.

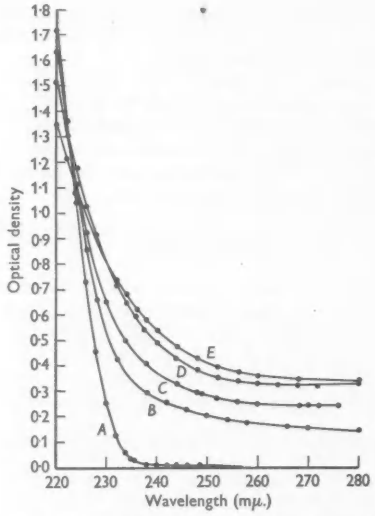


Fig. 1. Ultraviolet absorption spectra of aqueous solutions of L-histidine hydrochloride hydrate (100 μg./ml.). Cathode-ray dosage: curve *A*, control (non-irradiated); curve *B*, 100,000 r.e.p.; curve *C*, 250,000 r.e.p.; curve *D*, 500,000 r.e.p.; curve *E*, 1,000,000 r.e.p.

Dale, Gray & Meredith (1949) have stated that if the action of ionizing radiation is indirect, one would expect the ratio of inactivation dose D (energy required to reduce the amount of solute to 37 % of the initial value) to concentration (C), which they defined as the specific inactivation dose (D/C), to be independent of concentration over a wide range. Table 4 gives the specific inactivation doses of histidine at four concentrations, as calculated from the data in Table 1. The calculations were made from the following equation

$$n = n_o e^{-D/D_o},$$

where n_o is the initial concentration of the solute, n is the concentration of the solute that survives a dose D, and D_o is the dose required to reduce the solute to 37 % of its initial concentration ($1/e$). The values in Table 4 show that the specific inactivation dose (D/C) is almost independent of concentration within the range of concentrations studied. The above observations demonstrate that the action of high-voltage cathode rays on aqueous solutions of histidine is indirect.

When the concentration of the solute is low, it is possible that an appreciable proportion of the total number of active radicals may recombine with one another rather than react with solute molecules. This situation will not arise in concentrated solutions and the activated radicals will have a greater chance to react with the solute molecules before they can recombine to form water. It is seen from Table 4

Table 4. *Decomposition of histidine by high-voltage cathode rays*

Concentration of solution, C (g./ml.)	Inactivation dose, D (r.e.p.)	Specific inactivation dose, D/C (r.e.p./g./ml.)
$1{\cdot}0 \times 10^{-4}$	$1{\cdot}34 \times 10^{5}$	$1{\cdot}34 \times 10^{9}$
$2{\cdot}5 \times 10^{-4}$	$3{\cdot}21 \times 10^{5}$	$1{\cdot}29 \times 10^{9}$
$5{\cdot}0 \times 10^{-4}$	$5{\cdot}69 \times 10^{5}$	$1{\cdot}14 \times 10^{9}$
$10{\cdot}0 \times 10^{-4}$	$11{\cdot}60 \times 10^{5}$	$1{\cdot}16 \times 10^{9}$

that the specific inactivation dose does show a trend towards increasing values with decreasing concentration of solute, indicating that there may be some loss of radicals either by recombination or in other ways when the more dilute solutions are irradiated.

Ionic yield of the decomposition of histidine. From the data shown in Table 1, the ionic yields over a range of concentrations have been calculated, and the values are presented in Table 5. In the calculation of ionic yields, 1 r.e.p. is considered to produce $1{\cdot}6 \times 10^{12}$ ion-pairs/g. of tissue (Dainton, 1948).

Table 5. *Ionic yields of the decomposition of histidine monohydrochloride by high-voltage cathode rays*

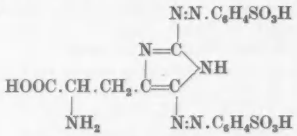
Concentration of solution (mg./ml.)	Dosage (r.e.p. $\times 10^5$)	Ionic yield M/N
0·1	1·0	1·00
	2·5	0·59
	5·0	0·34
0·25	1·0	2·14
	2·5	1·16
	5·0	0·64
	10·0	0·39
0·50	1·0	3·00
	2·5	1·40
	5·0	0·84
	10·0	0·64
1·0	1·0	3·40
	2·5	2·62
	5·0	1·29
	10·0	1·02

The overall ionic yields of histidine ranged from 0·34 to 3·40. At a given dosage the ionic yield increased with concentration. This observation is typical of radiochemical reactions, to which the indirect action theory may be applied and in which the amount of solute remaining unchanged is exponentially related to the dose. Lea (1947) considers that activated water is formed at the rate of 1 mol.

(one effective radical pair) per ion-pair and that reaction yields smaller than this are due to the proportion of collisions between reactant molecules and activated water leading to deactivation without reaction.

The data given in Table 5 demonstrate the high ionic yields in concentrated solutions. In view of the mechanism suggested by Miller (1948) and by Dainton (1948), the high ionic yields of the decomposition of histidine monohydrochloride can be explained by assuming the formation of several pairs of active radicals per ion-pair.

Evidence of rupture of the imidazole ring of histidine. In the colorimetric method used for the determination of histidine, colour develops on treating histidine with *p*-diazobenzenesulphonic acid. Pauly proposed the following structure for the coloured complex:



If the ring is broken, histidine cannot be estimated by this method.

Inspection of Table 2 shows that the residual amino-acid, as measured colorimetrically, diminished progressively with increasing dosage of irradiation. A comparison of these values with those obtained microbiologically shows that the microbiological values are lower than the chemical values in every case. This suggests that the microbiological assay measures the change both in the ring part of the molecule and the side chain containing the α-amino group.

Table 3 shows the yields of ammonia liberated from histidine solutions containing 100 μg./ml. Upon irradiation with a dose of 1,000,000 r.e.p., 11·23 μg. of NH_3/ml. were obtained. Microbiological assay showed in every case that no amino-acid was retained at this dosage of irradiation. If only the α-amino group were split off, 100 μg. of histidine monohydrochloride hydrate (mol.wt. = 209·64) would give rise to 8·11 μg. of ammonia, whereas 11·23 μg. of NH_3/ml. were obtained. This signifies that ammonia nitrogen is formed from the nitrogen atoms present both in the ring and in the side chain. This is further evidence that the imidazole ring of histidine is broken upon irradiation with cathode rays.

SUMMARY

1. Aqueous solutions of histidine monohydrochloride containing 100, 250, 500 and 1000 μg./ml. were irradiated with high-voltage cathode rays at four levels, namely, 100,000, 250,000, 500,000 and 1,000,000 r.e.p. Histidine was decomposed at all concentrations, and the decomposition was related exponentially to the dosage of irradiation.

2. The ionic yields ranged from 0·34 to 3·4 over the range of concentrations studied.

3. Data are presented to show that the action of high-voltage cathode rays on histidine is indirect.

4. It is concluded that cathode rays decompose histidine through deamination of the α-amino group and the fission of the imidazole ring.

We wish to thank Prof. J. G. Trump and Mr K. A. Wright of the Department of Electrical Engineering, Massachusetts Institute of Technology, for their co-operation in placing the electrostatic generator at our disposal for this study.

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The Action of Organic Acids on some Fibrous Proteins: the Oxidation of Wool Keratin

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Information as to the arrangement of amino-acid residues along the peptide chains of wool has been obtained from the study of the peptides present in partial acid hydrolysates (Consden, Gordon & Martin, 1949; Consden & Gordon, 1950). The present observations arose out of experiments designed to study in what way the individual peptide chains of wool differ from one another in amino-acid composition. The chains were separated by oxidizing the cystine cross-linkages to cysteic acid with performic acid, a technique originally applied by Sanger (1949) to insulin.

The action of formic acid and acetic acid on a number of fibrous proteins, including wool, has also been studied. Toennies (1942) has previously pointed out that many proteins are soluble in formic acid, which has been shown to cause wool fibres to increase in diameter by 50% (Speakman, 1931). Meyer & Mark (1930) showed that formic acid caused marked swelling of silk and a change in the X-ray diagram; these could be reversed on washing with water. From the present results it seems likely that the extent of solution of fibrous proteins in formic acid can be correlated with the molecular configuration of the peptide chains.

Alexander & Earland (1950*a*) found that, when wool which had been oxidized with peracetic acid (Alexander, Hudson & Fox, 1950) was treated with dilute aqueous ammonia, a fraction amounting to 7–10% by weight of the original wool remained insoluble. They considered this to be the 'sub-cuticle membrane' of the wool fibre and found it, on hydrolysis, to yield all the amino-acids obtainable from wool, but in different proportions. Mercer, Lindberg & Philip (1949) have contested the view that such a 'sub-cuticle membrane' or 'subcutis' exists, considering the preparations to consist of the thin 'epicuticle' membrane, thickened by the adhesion of other layers of the cuticle. Experiments bearing on the structure of this 'sub-cuticle membrane' are described by the present authors, the general conclusion being that the membrane does not as such exist in the original fibre.

The paper also records further observations on the action of alkaline hydrogen peroxide on wool (cf. Consden & Gordon, 1950).

MATERIALS AND EXPERIMENTAL PROCEDURES

Analytical methods

N determination, micro-Kjeldahl; total S, Barritt (1934); cystine and cysteine S, Shinohara (1935, 1936); partition chromatography on paper, Consden, Gordon & Martin (1944). Hydrolysis of proteins was effected by refluxing in 5N-HCl for 24 hr. and removing excess acid by repeated evaporation *in vacuo*. In certain cases hydrolysed protein was oxidized with H_2O_2 before running on a phenol chromatogram. Cystine originally present is thus oxidized to cysteic acid, which is then readily detected on the chromatogram (Dent, 1948).

Protein preparations studied

A 70's quality wool, commercially scoured and made into dry-combed top. (Found: total S, had 3·77%; (cystine + cysteine) S, 3·28%.) This wool was used throughout the present experiments unless otherwise stated. The silk fibroin was an unweighted fabric. Before use both were extracted with light petroleum (b.p. 40–60°), then with ethanol, rinsed in several changes of distilled water and dried in air. Collagen was an acetone-dehydrated, untanned hide.

Performic acid oxidation of wool

Wool (1 g.) was treated at room temperature with a mixture of 36 ml. of formic acid (98–100%) and 4 ml. of 30% (w/w) H_2O_2 for a given time (cf. Sanger, 1949), filtered off on sintered glass, rinsed well in water and dried. The dissolved protein was isolated either by evaporation *in vacuo* below 40° or by precipitation on pouring into water. (The dissolved material is described as protein, even though it may differ from the original protein.)

Treatment of proteins with formic and acetic acids

The protein (1 g.) was treated at room temperature with either formic acid (40 ml.; 30 ml. in the case of silk fibroin) or acetic acid (40 ml.) for a suitable time and filtered off. The protein in solution was examined after evaporating the acid *in vacuo* below 40°. Its amino-acid composition was examined on a two-dimensional collidine-phenol-NH_3 chromatogram after HCl hydrolysis.

A number of chemically modified wools were also treated with formic acid. Samples of wool were boiled under reflux for 1 hr. with 100 times their weight of solutions of different pH value, rinsed in water and dried. They were then treated with formic acid for 2 hr., the dissolved protein being estimated by determining N in the filtrate.

Modification of the surface of wool

Some experiments, described below, were carried out with wool whose surface layer had been modified chemically in one of two ways:

(*a*) Treatment by gaseous chlorination (King & Galley, 1933), in which 1·3% of Cl_2 was found to have reacted with the wool; only the surface layer of the wool is affected by the Cl_2 under these conditions (Phillips, 1942).

(*b*) Treatment by papain following the gaseous chlorination (Phillips, Middlebrook & Higgins, 1942).

The chlorinated wool was treated for 1 hr. at 60° with a solution containing 1% (w/v) of $NaHSO_3$ and 0·025% of papain, and adjusted to pH 5; 30 ml. of solution were used to each gram of wool. The treated wool was then milled in the stocks. Under these conditions the scales of the wool are removed almost completely. Both wools were extracted with solvents and rinsed in water before use.

RESULTS

Performic acid oxidation of wool

Wool treated with performic acid swells greatly and partially dissolves. Table 1 records the cystine sulphur of the undissolved protein when the wool had been treated for different times. Oxidation of the cystine was essentially complete in 30 min.; Sanger (1949) found that the cystine of insulin was completely oxidized in 15 min. The amount of protein going into solution, however, determined by weighing the undissolved residue, increased with increasing time of treatment (Table 1). The rate at which protein went into solution varied somewhat from experiment to experiment.

Table 1. *Oxidation of wool with performic acid*

(For conditions of experiment see text.)

Time of treatment (hr.)	Loss in weight (%)	Cystine S of undissolved wool* (%)
0·25	—	0·22
0·5	—	0·15
1	7	0·15
2	16	0·08
3	27	0·05
5	27	0·04
18	39	—

* The cysteine S was in every case less than 0·03%.

It appears that after oxidation of the cystine residues the wool keratin will dissolve in the large excess of formic acid which is present in the reaction mixture. From the data in Table 1, however, it is seen that the rate of solution decreases markedly after 5–6 hr. Wool which had been oxidized with performic acid for 2 hr., rinsed in water and air dried dissolved only slowly in formic acid at room temperature, protein equivalent to 9 and 6% of the original weight of the wool being dissolved during two successive treatments with fresh formic acid for 2 and 17 hr. respectively.

The X-ray diffraction patterns of the dissolved protein (from wool oxidized with performic acid for 0·5 hr., isolated by pouring into water) and of the undissolved wool were examined by Dr T. C. Tranter. The dissolved protein showed rings corresponding to spacings of 11·3 and 4·6 A., probably corresponding to the 'side chain' and 'backbone' spacings of a disordered β-keratin structure. The undissolved wool gave a weak photograph with two rings corresponding to spacings of 9·9 and 4·3 A. The spacings could only be considered as approximate in view of the weakness of the photograph, but they probably also correspond to a disordered β-keratin structure. The undissolved wool, therefore, has a similar X-ray structure to the dissolved protein as isolated. It does not, however, necessarily follow that the dissolved protein has the β-keratin structure while in solution.

The relatively slow dispersion of the oxidized wool in formic acid may be correlated with the assumption of the β-configuration. When the cystine cross-linkages have been broken by the action of the performic acid, the polypeptide chains are freed from restraint and can assume new configurations. Elongation of the chains takes place and the β-configuration is attained. Proteins in the β-configuration often offer a greater resistance to dispersion than those in the α-configuration (Blackburn, 1948).

The action of organic acids on fibrous proteins

The amount of wool keratin or silk fibroin dissolved by glacial acetic acid at room temperature is inappreciable, but some 6·6% of collagen dissolves during 48 hr. Formic acid has a more drastic action: silk fibroin and collagen swell greatly in it, much more than wool, and dissolve to an appreciable extent (Table 2). That formic acid has a greater solvent power for proteins than has acetic acid may also be seen from the appreciable solution of wool

Table 2. *Action of formic acid on fibrous proteins*

Protein	Time of treatment (hr.)	Protein dissolved by formic acid (%)
Wool (70's top)	2	0·34
	16	0·42
	48	0·45
Blackface wool	2	0·43
	24	0·56
Porcupine quill	72	7·0
Silk fibroin	0·5	10·6
	1	11·2
	2	11·3
	5	11·8
	24	12·8
	48	14·4
Collagen	72	23·1

which occurs during performic acid oxidation, as compared with that occurring during peracetic acid oxidation. Table 3 shows the amount of protein dissolved when silk fibroin was treated with formic acid for 3 days, removed and treated with a further amount of formic acid, the process being repeated thrice in all. The experiment was terminated at this point as the formic acid solution acquired a purple colour, possibly caused by degradation of tryptophan residues. The extent of the colour formation when collagen and wool were similarly treated was much less, but their tryptophan content is lower. It appears that formic acid will not readily dissolve all the silk fibroin, only some 20 % dissolving rapidly.

Table 3. *Repeated treatment of silk fibroin with formic acid*

(For experimental conditions see text.)

	Time of treatment (hr.)	Total protein dissolved by formic acid (%)
First treatment	72	14·6
Second treatment	48	17·5
Third treatment	48	21·2

In contrast, only relatively small amounts of wool keratin are dissolved by formic acid (Table 2). It seemed probable that the low solubility of wool keratin in formic acid might be due to the presence of the cystine cross-linkages. Wool in which the cystine has been reduced to cysteine shows a relatively high solubility in formic acid. When wool (1 g.) was reduced with 20 ml. of a 5·5 % (w/v) solution of thiolacetic acid at 39° for 17 hr. (cf. Patterson, Geiger, Mizell & Harris, 1941), rinsed in water, pressed between filter papers and treated with formic acid for 16 hr., a loss in weight of 25 % was observed. The action of formic acid on a number of keratins containing medulla, which has a low cystine content (Bekker & King, 1931; Blackburn, 1948), was also investigated. Only a slightly larger amount of protein was dissolved from a medullated Blackface wool than from the 70's top wool, but a considerably larger amount of porcupine quill was dissolved.

Table 4. *Action of formic acid on chemically modified wool*

pH of buffer solution	Wool dissolved by formic acid (%)
1·2 (Approx. 0·1 N-HCl)	0·72
3·0 (Table 62)*	0·40
4·8 (Table 70)	0·26
6·6 (Table 73)	0·23
8·2 (Table 79)	0·23
8·9 (Table 79)	0·29
10·0 (Table 85)	0·37
10·8 (Table 86)	0·49

* Buffers are cited in tables by Britton (1942).

Chemical modification of wool in which the cystine cross-linkages are not broken does not appear to alter its solubility in formic acid to any great extent. Thus, when wool is boiled in either acid or alkaline solution, the solubility in formic acid shows only a small increase (Table 4), which is probably to be attributed to some hydrolysis of the peptide chains.

The structure of the 'sub-cuticle membrane'

The introduction of the strongly acidic cysteic acid groups into wool keratin brings about a greatly increased solubility in alkali. The protein dissolved by performic acid and the insoluble wool residue are almost completely dissolved by dilute sodium carbonate or sodium hydroxide solutions at room temperature, and reprecipitated when the solution is acidified. When wool oxidized with performic acid for 0·5 hr. is treated with dilute ammonia (0·1–1·0 N) a greater proportion of the wool remains undissolved. Under the microscope this undissolved residue appears to be similar to the material described by Alexander & Earland (1950*a*, *b*). Chromatograms which were run on hydrolysates of the material insoluble in N- or 0·1 N-ammonia showed no marked differences from those of ordinary wool, except that there was a strong cysteic acid spot.

If, as Mercer *et al.* (1949) contend, the 'sub-cuticle membrane' preparations consist primarily of epi-cuticle with adhering layers of cuticle, modification or removal of the cuticle should make it difficult or impossible to isolate material with the same properties as the 'sub-cuticle membrane' from this treated wool. On the other hand, if the sub-cuticle membrane is a layer below the scales, removal or modification of the scales might have little effect on the isolation of the membrane. Experiments of this nature were therefore made to obtain further evidence on the question. Two treated wools, i.e. wool which had been chlorinated in order to modify the surface layer and wool which had been chlorinated and then treated with papain as described above to remove the scales together with the corresponding intact wool, were treated with peracetic acid followed by 0·1 N-ammonia under the conditions of Alexander & Earland (1950*a*). An insoluble residue, similar in appearance under the microscope to the preparation described by Alexander & Earland (1950*a*), was obtained in each case.

The amino-acid composition of the dissolved proteins

The protein dissolved by the performic acid treatment (0·25 or 0·5 hr.) of wool was examined on a two-dimensional collidine-phenol-ammonia chromatogram after hydrolysis. The chromatogram differed from that of normal wool in that a very strong cysteic acid spot was present and no tyrosine spot was observed. The dissolved protein from

samples of human hair, 56's top wool and the root ends of Cotswold wool fibres which had been oxidized with performic acid for 0·5 hr. was also isolated, hydrolysed and examined chromatographically. The chromatograms were similar to those of the intact proteins, except that once again cysteic acid was present and tyrosine absent.

The dissolved protein from the 3 hr. treatment of wool gave a result similar to that from the shorter treatment, but two additional spots were observed which ran fast in collidine and had R_F about 0·5 in phenol. The absence of tyrosine suggested that these additional spots might arise from the oxidation of tyrosine residues; tyrosine oxidized with performic acid gave rise to several spots in the same region of the chromatogram. The changes in colour of the wool which remained undissolved by the performic acid may also be correlated with changes in the tyrosine, though degradation of tryptophan residues may also play a part. After 0·5 hr. treatment the residue had darkened considerably, but after longer treatment it again became white in colour. Sanger (1949) found that certain of his fractions from oxidized insulin tended to darken on standing, suggesting the presence of oxidation products of tyrosine (cf. also Sanger & Tuppy, 1951). The possibility of some oxidation of threonine residues occurring cannot be completely ruled out.

The amino-acid composition of the soluble protein obtained when wool which had been reduced with thiolacetic acid was treated with formic acid did not differ greatly from that of normal wool, although the cystine (or cysteine) concentration was somewhat greater (peroxide test).

The dissolved protein obtained when collagen was treated with either acetic acid or formic acid, or when silk fibroin was treated with formic acid, appeared in each case to have a similar amino-acid composition to the corresponding intact protein.

The amino-acid composition of the small amount of wool dissolved by formic acid differed from that of intact wool, however, in that the amount of threonine in it was very low. Small amounts of cysteic acid were present, which did not increase appreciably after peroxide oxidation, indicating that very little cystine was present. The dissolved protein may, therefore, consist of peptide chains which have been liberated from the keratin structure by the splitting of cystine cross-linkages during the time the wool was exposed on the sheep's back (Race, Rowe, Speakman & Vickerstaff, 1938). Cysteic acid is known to be present in the tips of wool fibres which have suffered damage in this way (Consden, Gordon & Martin, 1946). The amino-acid composition of the dissolved protein from a medullated Blackface wool was similar to that obtained from 70's top, cystine and threonine being present in low concentration only. Cysteic acid was present.

When a lock of Lincoln wool was cut into three parts, degreased and treated with formic acid for 2 hr., the amounts of protein dissolved from the root, middle and tip portion were 0·26, 0·31 and 0·20% respectively. It might have been expected that more protein would be dissolved from the tip than from the root portions of the fibre, the former suffering greater damage during exposure to the weather. A possible complicating factor, however, is the presence of a certain amount of medulla in this Lincoln wool.

No peptides giving a colour with ninhydrin were revealed by running chromatograms on the (unhydrolysed) dissolved protein.

The dissolved protein from porcupine quills differed in amino-acid composition from that of the cortex, the concentration of glutamic acid, glycine, leucine and tyrosine being higher and that of the threonine lower. The dissolved protein was probably derived principally from the medulla, a similar chromatogram being obtained from medulla dissected out of the quill and treated with formic acid.

When wool was oxidized with hydrogen peroxide in alkaline solution, a polypeptide material went into solution. In this experiment 1 g. samples of wool were treated at 50° for times of 0·5, 1, 1·5, 2, 3, 4 and 5 hr., with 50 ml. of 15% (w/w) aqueous hydrogen peroxide brought to pH 9·17 by the addition of ammonia. The solutions were then decanted from the bleached wool and acidified with dilute hydrochloric acid. The white precipitate of polypeptide nature which settled out increased in amount with increasing time of treatment, and after 5 hr. amounted to 18·7 mg.

A collidine-phenol-ammonia chromatogram of the hydrolysed precipitate showed the usual amino-acids present in a wool hydrolysate, but the threonine spot was very weak and a strong cysteic acid spot was present. No cystine spot could be seen. Cysteic acid as well as cystine was present in the undissolved part of the wool.

DISCUSSION

Wool keratin, like insulin, is readily oxidized by performic acid. The oxidized keratin, however, shows no sharp separation on the basis of solubility into two fractions differing in amino-acid composition as did oxidized insulin (Sanger, 1949). The evidence from the present experiments, therefore, suggests that the peptide chains of wool do not differ markedly from one another in amino-acid composition. Some evidence that certain of the peptide chains have a lower threonine content than others is derived from the experiments in which wool was treated with alkaline hydrogen peroxide or with formic acid. However, only a small percentage of the wool was dissolved in these experiments. That there is some difference in position of the amino-acids

along the different chains is also made probable by the fact that the chains carry many different end groups (Blackburn, 1950; Middlebrook, 1949).

When proteins were treated with formic acid no marked differences were observed between the protein which went into solution and that which remained undissolved, and it is therefore improbable that the observed differences in solubility can be attributed to the presence of different fractions in the fibre. The results probably provide an instance of the chemical reactivity of a protein depending on its molecular configuration. Recent work has shown, for example, that the solubility of a long-chain polypeptide is dependent on whether it is in the α- or β-configuration (Bamford, Hanby & Happey, 1950).

Proteins in the β-configuration seem generally to disperse only slowly in the reagents used, e.g. silk fibroin in formic acid. On the other hand, wool keratin resists solution due to the presence of the cystine cross-linkages. After these have been broken the solubility increases, but is then dependent on the configuration of the peptide chains. The initial rate of solution of the protein having the α-configuration is fairly high, but as swelling of the remaining undissolved protein occurs the peptide chains elongate and when they have attained the β-configuration, in which they can lie closer together, the rate of solution diminishes. An example is the treatment of wool with performic acid. Another example may be the solution of wool in alkaline thiolacetic acid (Goddard & Michaelis, 1934). In this reaction, according to observations in these laboratories, some 40–50% of the wool generally remains undissolved. A further instance may be the solution of keratins in solutions of urea and sodium bisulphite (Jones & Mecham, 1943). The soluble protein in both these reactions resembles the intact wool in amino-acid composition.

The action of aqueous ammonia on wool oxidized with peracetic acid may well be another example of the same phenomenon. Alexander & Earland (1950*b*) and Happey (1950) found that the oxidized wool gave an α X-ray diagram, whereas the 'subcuticle membrane' prepared from it by treatment with aqueous ammonia had a β X-ray diagram. They considered the 'membrane's' resistance to attack to be due to its β form, where more intermolecular hydrogen bonds were present than in the α form. It is possible, however, that the β form arises during the alkali treatment of the oxidized wool and is not present as such in the original fibre.

The present experiments lend little support to the idea that the membrane forms a particular structural component present as such in the original fibre. The formation of similar structures from chlorinated and 'chlorzymed' (chlorinated and papain-treated) wool shows that the presence of the 'epi-cuticle' on the fibre is not essential to the isolation of the 'subcuticle' membrane (cf. Mercer *et al.* 1949). On the other hand, if the present explanation of the formation of this insoluble residue is correct, we should expect to, and indeed do, obtain a residue from chlorinated and papain-treated wool in the same way as from initially intact wool.

Peracetic acid oxidizes wool more slowly than performic acid, probably due to a slower rate of diffusion into the fibre occasioned by the smaller extent of swelling (cf. Alexander *et al.* 1950; Speakman, 1931). The still slower rate of oxidation by hydrogen peroxide in aqueous solution may similarly be related to the still smaller degree of swelling.

The product of the reaction in aqueous solution embodies oxidation products of the cystine residues at the disulphoxide or sulphinic acid stages (Harris & Smith, 1937; Consden & Gordon, 1950). The peptide material found in solution after the alkaline peroxide treatment of wool probably represents those peptide chains which have been set free from the structure by the dismutation of the residues of disulphoxide in the alkaline solution. The results of the chromatographic analysis of this peptide material after hydrolysis with hydrochloric acid are in accordance with this interpretation. Cysteic acid was shown to be present, but neither the disulphoxide nor the sulphinic acid from cystine-cysteine could be detected.

SUMMARY

1. The cystine of wool is rapidly oxidized by treatment with performic acid. The oxidized wool subsequently dissolves slowly and incompletely in the reagent solution. When wool is oxidized with alkaline hydrogen peroxide a small amount of long-chain peptide material goes into solution.

2. Fibrous proteins partially dissolve in formic acid at room temperature; the extent of solution has been correlated with the molecular configuration of the peptide chains of the protein. The small amount of wool keratin which is dissolved by formic acid probably consists of peptide chains which have been liberated from the structure by the action of weather on the raw wool.

3. These results are interpreted in terms of changes from the α-keratin to the β-keratin configuration of peptide chains present.

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Cytochrome *b* and Bacterial Succinic Dehydrogenase

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Bach, Dixon & Zerfas (1946) pointed out that the dehydrogenases fall into distinct groups according to the intermediate hydrogen acceptors through which they react with oxygen. Succinic dehydrogenase and lactic dehydrogenase of yeast come into the category of 'cytochrome-reducing dehydrogenases' because they readily reduce cytochrome *c*, and do not depend on coenzyme I or II for the reduction of methylene blue (Mb). None of the enzymes of this group of 'cytochrome-reducing dehydrogenases' has been isolated. Bach *et al.* (1946) purified the lactic dehydrogenase of yeast to a considerable extent and found that a new cytochrome, b_2, was concentrated in the enzyme fractions. There was an apparent correlation between the strength of the absorption band and the enzyme activity. They presented evidence that cytochrome b_2 forms an essential part of the enzyme system, but were not able to determine whether cytochrome b_2 is the dehydrogenase itself or an essential intermediate carrier between lactate and methylene blue. They also pointed out that in the heart muscle, cytochrome *b* is intimately associated with succinic dehydrogenase as shown by Keilin & Hartree (1939), and therefore it is possible that enzymes of this group are of the *b* type.

Ball, Anfinsen & Cooper (1947) state that the function of cytochrome *b* is unknown, and consider that it may be identical with that of succinic dehydrogenase.

In a study of the relationship between the iron enzymes of *Corynebacterium diphtheriae* and diphtheria toxin, Pappenheimer & Hendee (1947) obtained evidence that cytochrome *b* was directly concerned in the oxidation of succinate. They were unable to separate succinic dehydrogenase from cytochrome *b* by differential centrifugation. Pappenheimer & Hendee (1949) made a comparison between the diphtherial succinoxidase system and that of ox-heart muscle, and concluded that succindehydrogenase may well be identical with cytochrome *b* both in diphtherial extracts and in ox-heart preparations.

Slater (1949), working with heart-muscle and kidney tissue, found that cytochrome *b* was involved in the reduction of methylene blue by succinate. Slater was not able to decide whether cytochrome *b* is identical with succinic dehydrogenase or whether

cytochrome *b* is an enzyme additional to succinic dehydrogenase required for the reduction of methylene blue.

If cytochrome *b* is essential for succinic dehydrogenase activity it is obvious that one would not expect to find dehydrogenase activity without cytochrome *b*. The finding of such activity in the absence of the other would be proof of their non-identity.

A strictly anaerobic micrococcus, identified as *Veillonella gazogenes*, was isolated from the rumen of sheep (Johns, 1949). This micrococcus showed both succinic and lactic dehydrogenase activity, and as strictly anaerobic bacteria have not been reported as containing any of the cytochrome components (Stephenson, 1949) it was of interest to examine this organism for cytochrome bands and at the same time measure both lactic and succinic dehydrogenase activity.

METHODS

A very weakly catalase-positive strain of *V. gazogenes* was grown anaerobically in liquid media consisting of a general-purpose salt mixture (Stephenson, 1949) with 0·4% (w/v) Difco yeast extract and 1% (w/v) sodium lactate. Good growth was obtained in 16 hr. The culture was centrifuged down and washed twice in distilled water containing 0·01% (w/v) Na_2S.

Succinic and lactic dehydrogenase activity were determined by the Thunberg technique.

A portion of the bacterial suspension was placed in a wedge-shaped cell, frozen and cooled in liquid air according to the method of Keilin & Hartree (1949). It was examined with a direct vision hand spectroscope using a 250 W. projector lamp as a source of light.

RESULTS AND DISCUSSION

Keilin & Hartree (1949) have shown that freezing and cooling to very low temperature sharpens and intensifies the absorption bands of certain pigments, making possible the detection of cytochrome in cells where the pigment has not hitherto been seen. Examination of a suspension containing 34 mg. dry wt./ml. of bacteria with the addition of a little sodium dithionite revealed no absorption bands. When dilute sodium hydroxide, pyridine and hydrosulphite were added to a bacterial suspension at air temperature, there was no sign of the bands of a pyridine haemochromogen.

Part of the same suspension was used for dehydrogenase determinations. The results are given in Table 1.

Table 1. *Succinic and lactic dehydrogenase activity of a washed suspension of* Veillonella gazogenes

(Thunberg tubes contained 1 ml. bacterial suspension (21 mg. dry wt./ml.), 1 ml. 0·0002 M-methylene blue, 2 ml. 0·1 M-phosphate buffer, pH 7. Tubes evacuated and filled with N_2. Temp. 37°.)

	Contents of side arm (1·0 ml.)		
	0·1 M-Sodium succinate	0·1 M-Sodium lactate	Water
Reduction time (sec.)	30, 31	34, 30	No reduction in 2 hr.

Defining Q_{Mb} as the amount of methylene blue (in μl. H_2) reduced/mg. dry wt. of bacteria/hr. Q_{Mb} succinate = 25·6; Q_{Mb} lactate = 24·0.

A second experiment, similar to that shown in Table 1, was carried out with the addition of sodium malonate. The reduction of methylene blue in the presence of succinate was completely inhibited by 0·2 M-malonate, and 50% inhibition was achieved by a concentration of 0·025 M. Malonate is a competitive inhibitor of succinic dehydrogenase and this indicated that we were dealing with the reaction succinate + Mb → fumarate + Mb.H_2. Attempts to prepare an active cell-free solution of the succinic dehydrogenase were unsuccessful.

The evidence presented indicates that cytochrome *b* could not be detected in the intact cells of *Veillonella* even with the low-temperature technique, although the organism contained succinic and lactic dehydrogenases.

Bach *et al.* (1946) did not observe cytochrome b_2 in intact yeast. It was only after lactic dehydrogenase had been extensively purified that the two new absorption bands appeared. *Veillonella* may contain cytochrome b_2, but the methods used could not detect it. *Veillonella* strains vary considerably in their catalase content, and the one used in the present investigations showed only very slight activity when tested as a thick suspension. It was not present in sufficient concentration to show bands of pyridine haemochromogen at air temperature.

SUMMARY

Cytochrome bands could not be detected in an obligately anaerobic bacterium, *Veillonella gazogenes*, which contains succinic and lactic dehydrogenases.

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